University of Alberta

CYCLIC AMP AND PKA ARE ESSENTIAL FOR NEURITE OUTGROWTH IN RAT MOTONEURONS

by

Conrad Aglah

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ABSTRACT

Axon outgrowth and extension are essential requirements during normal development and in axonal regeneration of the adult nervous system. Cyclic AMP (cAMP) has been shown to promote survival of motoneurons *in vitro* in the absence of peptide growth factors but its role in the outgrowth and extension of motor axons is not known. We investigated the role of intracellular cAMP and its downstream target protein kinase A (PKA) on neurite outgrowth of motoneurons *in vitro*. Treatment of motoneurons with adenylyl cyclase inhibitor SQ22356 reduced intracellular cAMP and outgrowth and extension of neurites. Conversely, treatment with agents that elevate intracellular cAMP levels, forskolin, dibutyryl cAMP, 3-Isobutyl-1-methylxanthine and Rolipram increased the number of motoneurons that extended neurites and the length of the longest neurites. The cAMP-mediated effects on neurite extension and growth required PKA activation because the PKA inhibitors H89 and RpcAMP reduced neurite outgrowth. However the agents that elevated cAMP and promoted neurite outgrowth did not activate extracellular regulated kinases (Erk) which are essential components of the well-documented pathway for neurite outgrowth signaling. These data demonstrate an essential role of cAMP and PKA in the axonal growth and extension of motoneurons.

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ABBREVIATIONS

Arg 1:	Arginase 1
BDNF:	Brain Derived Neurotrophic Factor
cAMP:	Cyclic adenosine monophosphate
CNS:	Central Nervous System
CREB:	cAMP response element binding protein
DbcAMP:	Dibutyryl cAMP
DRG:	Dorsal Root Ganglion
Erk:	Extra-cellular regulated kinase
GAP-43:	Growth Associated Protein-43
GDNF:	Glial Derived Neurotrophic Factor
IBMX:	3-Isobutyl-1-methyl xanthine
MAG:	Myelin Associated Glycoprotein
NCAM:	Neural cell adhesion molecule
NgR:	Nogo Receptor
OMgp:	Oligodendrocyte Myelin glycoprotein
PDE IV:	Phosphodiesterase type IV
PKA:	Protein Kinase A
PNS:	Peripheral Nervous System
TrkA:	Tropomyosin receptor kinase-A
TrkB:	Tropomyosin receptor kinase-B

CHAPTER 1

GENERAL INTRODUCTION: AXONAL REGENERATION IN THE CENTRAL AND THE PERIPHERAL NERVOUS SYSTEM

1.1 INTRODUCTION

Both sensory and motoneurons of the peripheral nervous system (PNS) regenerate their axons after injuries and generally this capacity is contrasted with the inability of the central nervous system (CNS) injured neurons to regenerate their axons. This observation has been attributed to the growth permissive environment of PNS in contrast to growth inhibitory environment of adult CNS myelin. In the PNS Schwann cells respond in ways that ensure extensive axonal regeneration after injury. Functional recovery though is frequently disappointing (Fu and Gordon, 1997). In addition, macrophages help clear off debris of phagocytosed extracellular matrix after degeneration thereby promoting axonal regeneration. The inability of the CNS to regenerate is mainly attributed to the inhibitory molecules in CNS myelin and the extracellular environment including the glial scar. It is becoming clearer that the factors, on which axonal regeneration in both the PNS and the CNS depends, include not only the extracellular; growth promoting or inhibitory factors but also the intrinsic state of the neuron and its ability to respond to these factors.

1.1.1 Cellular responses to nerve injuries

For a successful axonal regeneration, the injured neuron must survive the injury and this survival depends on myriads of factors. The factors on which survival depends include the type of neuron, age, and the extent of the injury and the proximity of the injury to the cell body (Fu and Gordon, 1997).

In the PNS most adult sensory neurons and motoneurons survive when axotomized. Those that do not survive die presumably by apoptosis with the dead neurons displaying characteristic morphological changes associated with apoptosis and

DNA fragmentation (Lo et al., 1995). Evidence abounds to support the fact that neuronal death is caused in part by neurotrophic factor deprivation, though some axotomized motoneurons have been known to survive neurotrophic factor deprivation (Fu and Gordon, 1997). Neurons that survive axotomy undergo characteristic morphological changes termed chromatolysis. Chromatolysis is marked by characteristic changes in the neuronal cell body, which include dissolution of the ribosomes and the endoplasmic reticulum (Nissl bodies), cell swelling, eccentric positioning and enlargement of the nucleus. These changes lead to a marked increase in mRNA synthesis and a change in gene expression leading to switch from the normal mature "transmitting" mode of the neuron to "growth" mode (Fu and Gordon, 1997). The altered gene expression results in down-regulation and up-regulation of various proteins. These include expression of immediate early genes such as c-*jun* and *jun* B m RNA, microfilament proteins, enzymes, cytoskeletal proteins, and growth associated proteins including GAP-43 (Tetzlaff et al., 1991). Reduced expression of enzymes such as choline acetyltransferase and acetylcholine esterase, and synaptic proteins such as synaptobrevin (Friedman et al., 1995; Kishino et al., 1997) is consistent with a switch from "transmitting" to "regenerating" phenotype in axotomized neurons. The enzymes urokinase and plasminogen activators help digest the extracellular debris and clear the path for regeneration of the axotomised neuron (Muir et al., 1998; Siconolfi and Seeds, 2001). Expression of genes to support regeneration by axotomised neurons in the CNS is often suppressed. It is known that genes coding for protein components of axonal growth cones are suppressed in mature neurons but become readily activated when for example the peripheral branches of DRG neuron are injured. Evidence abounds that these growth-

associated genes in the majority of CNS neurons remained suppressed following injury (Fernandes et al., 1999).

1.1.2 Schwann cells in axonal regeneration

Because of their intimate developmental, biological and morphological relationships with axons, Schwann cells play a pivotal role in processes of axonal regeneration. Active Schwann cells together with fibroblasts and other non-neuronal cells respond to nerve injury by proliferation. Schwann cells that have been previously quiescent and suddenly deprived of axonal contact undergo mitosis and line up to form a band of Bungner along the basement membrane of the endoneurial tube which guides the regenerating axon (Fu and Gordon, 1997). Signals responsible for the Schwann cells response to axonal injury are not yet clearly understood. Macrophage invasion after nerve injury, release of Schwann cell mitogens by microphage digestion of myelin, and the growth of Schwann cells into the denerveted nerve stump are believed to be some of signals (Fu and Gordon, 1997). Schwann cells switch their mode to growth support at the distal nerve stump as the severed axon switches form transmitting to regenerating mode. There is a switch in gene expression of Schwann cells of both myelinating and non-myelinating types to dediffentiated denervated scwhann cell phenotype which is associated with upregulation of regeneration associated genes and down regulation of myelin-associated proteins. The denervated Schwann cells promote axonal elongation by increasing their synthesis of surface cell adhesion molecules (CAMs), such as N-CAM, Ng-CAM/L1, N-cadherin, L2/HNK-1 and integrins and by elaboration of extracellular matrix proteins such as laminin, fibronectin and tenascin (Fu and Gordon, 1997);

Expression of the proper adhesion molecules is necessary for successful regeneration with integrins known to play an important role in regulating cell motility through focal adhesion (Snider et al., 2002). Trophic factors such as brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), nerve growth factor (NGF), cytokines, as well as their receptors have also been produced by the denervated Schwann cells of distal nerve stump (Hoke et al., 2000) and are believed to support the injured and the regenerating axon.

1.1.3 Staggered and slow rate of axonal regeneration in the PNS

Despite the capacity of the PNS to regenerate, functional recovery is generally poor in association with a time-limited capacity of injured neurons to regenerate and for Schwann cells to support this regeneration (Fu and Gordon, 1995b, 1995a, 1997). It has been recently discovered that axon outgrowth and crossing from the nerve stump proximal to the injury into the distal nerve stump after nerve repair is staggered and rate limiting (Brushart, 1988; Fu and Gordon, 1997). This process of regeneration across the surgical repair site takes as long as 4 weeks in the rat. Axonal regeneration then proceeds at a rate of 3mm/day and allow for the progression of the axons within the distal nerve pathway. Attempt to enhance and to speed up the rate of this process of axonal regeneration have shown that e*lectrical stimulation* greatly accelerates axon outgrowth but not rate of regeneration (Al-Majed et al., 2000). This increased rate of axon outgrowth by electrical stimulation has been mimicked in recent investigation by subcutaneous delivery of the phosphosdiesterase inhibitor rolipram in concentrations that promoted regeneration of axons in the CNS, indicating that the rolipram like the electrical

stimulation accelerates this axonal outgrowth (Nikulina et al., 2004b; Furey and Gordon, 2005).

1.1.4 Cyclic (cAMP) in axonal growth and regeneration

Cyclic AMP was the first of many second messengers to be identified and studied and the research has provided a framework for the understanding of the many signal transduction pathways that regulate many physiological and cellular processes including inflammation, sensory signaling, neuronal plasticity and transcription in vertebrates (Beavo and Brunton, 2002). In the nervous system, the role of cAMP is immense in that it mediates many neuronal processes either directly or indirectly. These processes include neurite outgrowth (Neumann et al., 2002; Qiu et al., 2002a), neuronal differentiation (Liesi et al., 1983), modulation of growth cone responses to a range of diffusible and non-diffusible factors (Song et al., 1997; Song and Poo, 1999a), axonal guidance (Song et al., 1997), neuronal survival (Rydel and Greene, 1988) axonal growth and regeneration (Carlsen, 1983a; Carlsen et al., 1987), neuronal plasticity and memory (Barad et al., 1998).

Unlike PNS axons, CNS axons do not regenerate spontaneously due to myriads of factors. These factors are classified into two main groups; the astrocytic glial scar that forms during injury, and inhibitors found in myelin (Fawcett, 1997; Fawcett and Geller, 1998; Filbin, 2003; Silver and Miller, 2004). The astrocytic glial scar formed during injury presents not only a mechanical barrier to growth cones but also secretes putative inhibitory molecules including proteoglycans which inhibit axonal regeneration (McKeon et al., 1991). CNS myelin contains proteins that are growth inhibitory in a variety of

neurons (DeBellard et al., 1996). These proteins include myelin associated glycoprotein (MAG) (McKerracher et al., 1994), Nogo (Bandtlow and Schwab, 2000) and oligodentrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002). Another exciting discovery is that MAG, OMgp and Nogo exert their inhibitory effects via a common receptor Nogo 66 (NgR) (Fournier et al., 2001), or the receptor complex; the NgR/ $p75^{NTR}$ complex (Wang et al., 2002a; Wang et al., 2002b) and that the $p75^{NTR}$ transduces MAG inhibitory signals through Rho, a small intracellular GTPase (Niederost et al., 2002; Yamashita et al., 2002). The convergence of these inhibitory signals via the Rho pathway and the blockade of Rho activation by cAMP-dependent protein kinase A (PKA) (Higuchi et al., 2003) suggest intricately regulated mechanisms of axonal growth inhibition and regeneration in the CNS. The identification of the Nogo receptor (Fournier et al., 2001), cAMP effects (Cai and Filbin, 1999; Cai et al., 1999; Cai et al., 2001; Neumann et al., 2002; Qiu et al., 2002b) and the involvement of Rho pathway (Niederost et al., 2002; Winton et al., 2002b; Yamashita et al., 2002) in axonal growth and regeneration are encouraging indicators to finding therapies for CNS injuries. In particular, the role of cAMP in not only removal of myelin inhibition but promotion of neurite outgrowth are encouraging findings that could be exploited to speed up the otherwise slow and staggered axonal regeneration in the PNS.

1.1.5 EVIDENCE FOR THE INVOLVEMENT OF CYCLIC AMP IN AXONAL GROWTH AND REGENERATION

1.1.5.1 Cyclic AMP in nerve injury and regeneration

The first insight into the positive effects of cyclic AMP on axonal regeneration was provided by Roisen and colleagues who demonstrated from experiments that dorsal root ganglion neurons regenerated with increased length and numbers of axons when chick dorsal root ganglia were cultured with an analog of cAMP, dibutyryl cyclic AMP (dbcAMP) (Roisen et al., 1972). Also endogenous cyclic AMP was found to remain high for considerable period of time in regenerating nerve segments during sprout formation, elongation and axonal growth (Carlsen, 1982b, 1982c; Kilmer and Carlsen, 1984). Additional evidence in support of cyclic AMP involvement in nerve regeneration came from *in vivo* studies on regenerating axons of the crushed peripheral nerves. Chronic infusion of forskolin or either of the two more permanent analogs of cyclic AMP, dibutyryl cyclic AMP and 8-bromo cyclic AMP to the crush injured nerves *in vivo* reduced the time to initiation (latent growth period) of regenerative outgrowth and increased the rate of axonal elongation (Kilmer and Carlsen, 1984; Kilmer and Carlsen, 1987)

1.1.5.2 Cyclic AMP and the growth cone

The challenges of a growing axon during development or regeneration include finding the correct pathways and recognition of targets appropriate for its functions. Several guidance molecules and intracellular signaling pathways direct this complex system of

which cyclic AMP has been found to play a role (Tessier-Lavigne and Goodman, 1996; Song et al., 1997; Song et al., 1998; Song and Poo, 1999b). Decline in responsiveness of growing retinal ganglion axons to netrin-1, a guidance molecule that guide growing axons during embryogenesis has recently been demonstrated (Shewan et al., 2002). It was observed that netrin-1 receptor expression correlated with cAMP levels, such that a decline in cAMP concentration resulted in decreased responsiveness of the growing axons to netrin-1 (Shewan et al., 2002). Similarly, electrical stimulation was presumed to increase cAMP levels by activation of Ca^{2+} dependent adenylyl cyclase via voltage-gated channel dependent Ca^{2+} entry mechanisms modulates the response of growth cones to gradients of netrin-1 (Song et al., 1997; Ming et al., 2001). Ming and colleagues showed that a brief period of electrical stimulation converts repulsion of young *Xenopus* spinal neurons to attraction, and enhanced attraction in older cultures. In another experiment involving a gradient of soluble recombinant MAG (rMAG), the repulsive turning responses of the growth cone to MAG was converted to attraction by a brief period of electrical stimulation. The involvement of cAMP dependent pathways in the electrical stimulation of growth cone behavior were examined by adding to the culture of adenylyl cyclase inhibitor, SQ22356 which completely abolished turning behaviors induced by the stimulation, and thus confirming the involvement of cyclic AMP in growth cone guidance (Ming et al., 2001).

1.1.5.3 Cyclic AMP and the intrinsic growth capacity of the neuron

Axonal response to myelin, MAG and Nogo has been shown to differ with neuronal type and age (DeBellard et al., 1996; Bandtlow, 2003). Also MAG and myelin inhibit

regeneration of adult neurons but not embryonic neurons either in culture (Shewan et al., 1995) or when transplanted *in vivo* (Li and Raisman, 1993). Attempts to elucidate the molecular mechanisms underlying these observations pointed to a change in neuronal cAMP levels with development (Cai et al., 2001). These changes in cAMP levels with development thus regulate the response of the growing or adult axons to inhibitory factors, guidance cues and a range of diffusible factors (Song et al., 1997; Cai et al., 2001; Shewan et al., 2002). Cai and colleagues demonstrated that endogenous levels of cyclic AMP in three different types of neurons drops dramatically with development and that this developmental switch coincides with a switch to the inhibition of regeneration by MAG and myelin. Inhibition of older neurons by myelin, MAG and Nogo was prevented by neuronal cAMP elevation (Bandtlow, 2003). Similarly, inhibiting a signal downstream of cAMP activation (inhibition of PKA) in young neurons abolishes the early developmental promotion of axonal regeneration by myelin and MAG (Cai et al., 2001). Thus the inability of the adult CNS to regenerate is partly due to age related decline in cAMP levels which regulates Rho GTPase activity in growing axons.

1.1.5.4 Neurotrophic factors and cyclic AMP

It has also been found that the inhibitory effects of MAG and of CNS myelin could be overcome by application of neurotrophins (Cai et al., 1999). In their study, Cai and colleagues showed that priming neurons with neurotrophins such as brain derived neurotrophic factor (BDNF) and glial derived neurotrophic factor (GDNF) elevates neuronal cAMP level and overcomes axonal growth inhibition by MAG/myelin in a time- and dose-dependent manner. The mechanism proposed being that MAG/myelin mediate

inhibition via an unknown Gi protein which blocks increases in cAMP and that cAMP elevation activates PKA which removes this inhibition (Cai et al., 1999). This observation adds to the increasing complexity of the neurotrophin effects and signaling in the nervous system but yet not surprising as cAMP-neurotrophins mediate other cellular processes including inhibition of apoptosis, neuroprotection and enhancement of neuronal survival (Rydel and Greene, 1988; Hanson et al., 1998; Meyer-Franke et al., 1998). For example the survival and growth of embryonic spinal motoneurons, neonatal sympathetic and embryonic sensory neurons *in vitro* in the absence of neurotrophic support (Rydel and Greene, 1988; Hanson et al., 1998) and the responsiveness of dissociated retinal ganglion cells (RGCs) to peptide trophic factors (Meyer-Franke et al., 1995) is enhanced by intracellular cAMP elevation. Cyclic AMP and depolarization have also been shown to increase the recruitment of TrkB receptors to the plasma membrane of CNS neurons by translocation from intracellular stores (Meyer-Franke et al., 1998). Taken together, the survival, growth and regeneration effects of neurotrophins and/or via cAMP suggest signaling mechanisms or pathways that may involve cross-talks yet to be understood. Evidence that lend credence to this view was provided when it was shown that neurotrophins activate the extracellular regulated kinase Erk1/2 which then inhibits the phosphodiesterases type IV and as a result increase intracellular cAMP and promotion of neurite outgrowth (Gao et al., 2003).

1.1.5.6 Nerve conditioning lesion mediates regeneration via cAMP elevation

The regenerative capacity of the peripheral and the central branches of dorsal root ganglion neurons is known to be affected differently as a result of their different environments (Bradbury et al., 2000). Regeneration occurs readily when the peripheral branch is cut unlike the central branch after it had entered the CNS. When the peripheral axons are lesioned first, and the central axons cut 1 or 2 weeks later, significant growth is observed (Neumann and Woolf, 1999). Cyclic AMP elevation was subsequently shown to underlie the molecular basis of this observation, with effects, PKA-dependent and PKA independent (Qiu et al., 2002b). This observation was supported by Neumann and colleagues (2002) who made strikingly similar observations that confirmed the involvement of cAMP by using cAMP analogue dibutyryl cAMP (db cAMP) (Neumann et al., 2002). In both cases, a transient increase in cAMP on the prior peripheral conditioning lesion overcomes MAG and myelin inhibition and promotes regeneration in the CNS by two separate mechanisms; PKA-dependent (early phase) and PKA-independent (late phase) mechanisms. In the early phase, cAMP activation of PKA is presumed to have direct effects on the cytoskeleton and subsequently transcription whereas the late phase affects are noticed as the proteins synthesized via transcription during the early phase persist and affect growth directly thus PKA independent (Qiu et al., 2002b)

1.1.5.7 Downstream consequences of cAMP elevation in neurons

The central role of cAMP in regeneration prompted questions as to what are the main downstream pathways or consequences of cAMP actions that resulted in regeneration. The downstream consequences of cAMP elevation and activation of protein kinase A (PKA) that results in regeneration on myelin *in vivo* has been shown to be through gene transcription and up-regulation of the enzyme Arginase I, a rate limiting enzyme in polyamines biogenesis (Cai et al., 2002). Up-regulating Arginase 1 and/or application of polyamines mimic the effects of conditioning lesion and overcome MAG and myelin inhibition as demonstrated in earlier experiments confirming late phase PKA independent effect of cAMP (Cai et al., 1999; Cai et al., 2002). That the downstream consequences of elevated cAMP levels in neurons is the synthesis of polyamines comes as no surprise since polyamine levels have long been known to be increased during axonal injury and regeneration (Lindquist et al., 1985), an observation consistent with elevated cAMP levels observed in severed axons (Carlsen, 1982a). Polyamines regulate the neuronal cytoskeleton, enhance neuronal survival and accelerate the rate of axon regeneration and functional recovery after sympathetic and motor (sciatic) nerve injuries (Gilad et al., 1996). Though an exciting breakthrough it is yet to be determined the exact mechanisms of the signaling cascades involved in the actions of the polyamines.

1.1.6 Signaling axonal growth inhibition and regeneration: pathways regulated by cAMP

The understanding of the signaling cascades involved in axonal inhibition, growth and regeneration after injury is becoming clearer than ever. The main players involved:

the inhibitory molecules, for example, MAG, Nogo, OMgp and the proteoglycans; the receptors, Nogo66/NgR, p75 and the signaling molecules, Rho and cAMP seem converged and intricately regulated (Spencer et al., 2003). Recent evidence delineates the Rho signaling pathway as the convergence point via which all the inhibitory molecules of axonal regeneration exert their effects (Dubreuil et al., 2003). Thus, the inhibitory proteins whether NgR-dependent or NgR-independent signal their inhibitory effects via the Rho signalling pathway (Niederost et al., 2002; Winton et al., 2002a; Yamashita et al., 2002). Effects of Rho activation in neurons are well known which includes neurite retraction, cytoskeletal regulation, and cell rounding (Tigyi et al., 1996). The expression patterns of Rho after spinal cord injury (SCI) also correlates with the expression patterns of the inhibitory molecules in the CNS with Rho expression pattern being $p75^{NTR}$ dependent (Dubreuil et al., 2003). P^{75NTR} dependent apoptosis during SCI is attenuated by blockade of Rho activation (Dubreuil et al., 2003). In addition, inactivation of Rho and Rho kinase promotes regeneration and functional recovery after CNS injury (Lehmann et al., 1999; Dergham et al., 2002). On the other hand, cAMP elevation overcomes all the inhibitory molecules in the CNS and enhances axonal regeneration (Qiu et al., 2002a). The contrasting signaling roles of cAMP and Rho have been known for sometime now, albeit, poorly understood. It is therefore not surprising that these signaling pathways converge in regulating axonal inhibition, regeneration and growth. In mediating inhibition, the three major inhibitors in CNS myelin, MAG, Nogo, and OMgp mediate their effects via the Nogo 66 receptor (NgR) which has no transmembrane and cytoplasmic domains hence requires $p75^{NTR}$ as a co-receptor (Yamashita et al., 1999; Wang et al., 2002a) to transduce these signals. It is therefore apparent that axonal injury

triggers the release and expression of the inhibitory molecules which bind to NgR which then transduces the signals to the co-receptor $p75^{NTR}$ and consequently to Rho to cause axonal growth inhibition and neurite retraction. The action of cAMP on Rho is believed to be mediated via the cyclic AMP dependent protein kinase A (PKA). PKA activation regulates the activities of Rho GTPases, for example by blockade of the action of Rho via mechanisms that are not yet completely understood (Tigyi et al., 1996; Bandtlow, 2003). It has however been shown that cyclic AMP dependent protein kinase A phosphorylates RhoA at the carboxyl terminal portion on serine residue 188 to cause Rho inactivation in neuronal cell lines (Dong et al., 1998). This phosphorylation thus decreases the binding affinity of RhoA to its downstream effector Rho kinase alpha and subsequent ablation of the effects of RhoA activation. Phophorylation of Rho by PKA is not the only mechanism proposed for cAMP-PKA effects on Rho. In a recent study, Higuchi and colleagues have demonstrated the requirement of cAMP-PKA activation in translocation of the $p75^{NTR}$ to lipid rafts and the subsequent role of cAMP-PKA phosphorylation of $p75^{NTR}$ in regulating the biochemical and biological actions of the $p75^{NTR}$ such as Rho inactivation and neurite outgrowth by two main mechanisms; that is, RhoA is inactivated downstream of cAMP-PKA via phosphorylation and, that PKA by translocating $p75^{NTR}$ to lipid rafts, induce conformational changes which consequently lead to RhoA inhibition (Higuchi et al., 2003). The antagonism demonstrated by the cAMP and Rho pathways seems consistent with the roles of cAMP in overcoming the inhibitory effects of CNS myelin and its associated inhibitory molecules, regulation of growth cone guidance and promotion of axonal regeneration in the neurons of the CNS and the PNS (Song et al., 1997; Song et al., 1998).

1.1.7 Ras-Erk in axonal growth and regeneration

The Ras-Erk system is a principal signaling pathway involved in growth in many cellular systems. It is activated mainly via the growth factor receptor activation. However, recent evidence suggests activation via cross-talk with other signaling molecules. It is a well established signaling pathway that regulate neurite outgrowth in neurons (Kaplan and Miller, 2000). Previous evidence has established that elevation of cAMP can lead to activation of the Ras-Erk pathway in PC12 cells, hippocampal neurons (Grewal et al., 2000; Kiermayer et al., 2005), DRG neurons (Gao et al., 2003) and many other neuronal cell types (Stork and Schmitt, 2002). In addition dbcAMP has been shown to directly activate Erk to promote neurite outgrowth. An interesting example of the cross-talk between the Ras-Erk and the cAMP pathways is one demonstrated by Gao and collegues. In their work it has been established that treatment of DRG neurons with neurotrophins activate the Ras-Erk pathway such that activated Erk then phosphorylates and inhibit cAMP phosphodiesterases type IV (Gao et al., 2003). Phosphodiesterases degrade cAMP and their inhibition leads to elevated intracellular cAMP which then promotes neurite outgrowth.

1.1.8 Aim of this Study

The purpose of this study is to examine the role of cAMP and its downstream effector PKA in axonal growth in the PNS. Accordingly, in this study a rat motoneurons culture model was used and agents that modulate intracellular cAMP applied. Intracellular cAMP levels were measured and neurite outgrowth examined. Hitherto no work has been done with motoneurons in culture to investigate effects of cAMP on

neurite outgrowth. In addition, the motoneuron culture model provided a means to molecularly understand the mechanisms of cAMP effects on neurite outgrowth. The results of this study will shed light on whether raising intracellular cAMP in motoneurons pharmacologically (or via electrical stimulation) would help speed up axonal regeneration in the PNS which hitherto has been slow. In addition the study attempts to elucidate if Erk signaling pathways is involved in cAMP/PKA mediated neurite/axonal outgrowth.

CHAPTER 2

CYCLIC AMP AND PKA ARE REQUIRED FOR NEURITE OUTGROWTH IN RAT MOTONEURONS

2.1 INTRODUCTION

The ubiquitous intracellular mediator cAMP has been implicated in several neuronal processes and functions in different groups of neurons. Such functions include neuronal differentiation (Fujioka et al., 2004), axonal guidance (Song et al., 1997), neuronal survival (Hanson et al., 1998; Meyer-Franke et al., 1998), neurite outgrowth and axonal regeneration in the central nervous system (CNS) (Roisen et al., 1972; Cai et al., 1999; Neumann et al., 2002). Central axon regeneration is normally inhibited by CNS myelin-containing OMgp and MAG all of which bind to the common Nogo receptor to mediate axon growth inhibition (McKerracher and Winton, 2002). Elevation of cAMP and activation of the cAMP signaling cascade converts the repulsive turning of growth cones and inhibition of neurite outgrowth by these myelin associated inhibitors to attraction and neurite extension (Song et al., 1998; Cai and Filbin, 1999; Cai et al., 1999). The finding that high levels of cAMP in neonatal dorsal root ganglion (DRG) neurons permit neurite extension on CNS myelin, provided supportive evidence that cAMP plays a role in neurite outgrowth and/or axonal regeneration on a non-permissive growth substrate (Cai et al., 1999). Likewise, elevation of cAMP promotes neurite outgrowth of DRG neurons on permissive substrates (Neumann et al., 2002), consistent with *in vivo* evidence obtained in the 1980's for a role of cAMP in the regeneration of sensory nerve axons in the peripheral nervous system (PNS) (Carlsen, 1982b, 1983b).

Despite the capacity of axon regeneration in the PNS, functional recovery is frequently poor in association with reduced capacity for regeneration and reduced support of Schwann cells over time and distance (Acheson et al., 1995; Fu and Gordon, 1995b, 1995a, 1997). It has recently been established that axon outgrowth from proximal to

distal nerve stumps after mechanical nerve repair may require periods of up to a month, constituting an essential rate-limitation to successful axon regeneration and functional recovery; a brief period of electrical stimulation greatly accelerates the outgrowth (Al-Majed et al., 2000; Brushart et al., 2002). If indeed cAMP is involved in promoting neurite/axonal outgrowth, regulation of cAMP levels in neurons may constitute a practical method of promoting nerve regeneration in both the CNS and the PNS. The evidence obtained in the experiments of Filbin and her colleagues indicates that elevated neuronal cAMP levels and activation of protein kinase A (PKA) are pivotal to axon outgrowth in cerebellar and DRG neurons on a non permissive substrate (Cai and Filbin, 1999; Cai et al., 1999; Qiu et al., 2002b). In this study, we have purified motoneurons in order to determine whether cAMP plays a central role in neurite outgrowth on a permissive substrate and to establish the downstream molecular mediators of cAMP. The Ras-Erk pathway is one of the main pathways responsible for neurite extension (Stork and Schmitt, 2002; Pouyssegur and Lenormand, 2003; Dumaz and Marais, 2005; Chu et al., 2006) raising the question as to whether it is involved in cAMP mediated neurite outgrowth. Here we demonstrate that cAMP is required for neurite outgrowth in primary cultures of motoneurons. We show that neurite outgrowth is mediated via PKA but the Ras-Erk pathway is not activated downstream of cAMP-PKA.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Timed pregnant Sprague Dawley rats (day 15 pregnant) were obtained from the animal breeding facility at the University of Alberta and all procedures were approved by

the Animal Welfare Committee of the Institution. The timing of pregnancies was determined from the appearance of sperm plugs in the breeding cages. The morning of detection of the sperm plug was considered the day zero of the pregnancy.

2.2.2 Motoneuron culture

Establishment of pure motoneuron cultures was carried out as described (Arce et al., 1999; Raoul et al., 2005). In this procedure motoneurons from day 15 embryonic rats (E15) were dissociated and purified to greater than 90% homogeneity using an antibody against surface receptor $p75^{NTR}$ which is selectively expressed by embryonic motoneurons at day 14 -15 (Yan and Johnson, 1988).

2.2.2.1 Dissection of E15 rat embryos

Incision was made across the lower abdomen and rat embryos (E15) were delivered under halothane anaesthesia (1.25-1.5% delivered in 95% O_2 and 5% CO_2). The embryos were transferred into an iced-cold 1x phosphate buffered saline (1x PBS, pH 7.4) (Gibco) and the membranes surrounding the embryos removed. The embryo was placed the stomach on a silicone base support in a pyrex petri-dish containing PBS as above and the legs pinned under a dissecting microscope. Using forceps and a pair of scissors, the meninges were removed and the spinal cord lifted from the rostral end by holding the head towards the caudal end until the spinal cord is completely removed from the spinal column.

2.2.2.2 Preparation of suspension of dissociated spinal cord cells

Each spinal cord was cut into 15 pieces using a scalpel and fragments equivalent to 4 spinal cords transferred into a 15 ml polysterene tube containing 1ml complete neurobasal medium. 10µl Trypsin (Gibco, 2.5 % w/v; final concentration 0.025 % w/v) was added and incubated for 10 min at 37 °C with frequent agitation. The cell suspension was removed and fragments transferred into a 15 ml polysterene tube containing 800 µl complete neurobasal medium, 100 µl of BSA (4 % w/v in neurobasal medium) and 100 µl Dnase (1mg/ml in neurobasal medium). The fragments were vigorously shaken manually until disaggregated and then gently triturated twice. Fragments were allowed to settle for 2 min and the supernatant collected into another 15 ml polysterene tube. To the remaining fragments were added 800 µl of complete neurobasal medium, 100 µl of BSA (4 % w/v in neurobasal medium) and 100 µl Dnase (1 mg/ml in neurobasal medium); and triturated 8 times and allowed to settle for 2 min. The supernatant was collected and added to that collected earlier. If there were visible fragments present then the trituration step was repeated. The spinal cord suspension was then lowered on top of a 2 ml BSA (4 % w/v in neurobasal medium) and centrifuged for 5 min at 500 g. The supernatant was removed and spinal cord cell pellets resuspended in complete neurobasal medium. The dissociated cell suspension was then layered on top of density separation medium (Optiprep (Sigma) 1:5 dilutions in complete neurobasal medium) and centrifuged for 15 min at 800 g. The large cells containing motoneurons were removed from the top layer and diluted to 10ml with complete neurobasal medium in a 15 ml polysterene tube. The resulting suspension was then layered over 2 ml BSA (4 % w/v in neurobasal medium)

and centrifuged at 500 g for 5 min. After removing the supernatant the cell pellets consisting of large cells were then taken through the immunopurification procedure.

2.2.2.3 Immunopurification of motoneurons

Large cells consisting of motoneurons and other cells of the spinal cord collected from the surface of the top layer were incubated with mouse anti-p75 ^{NTR} antibody (MAB 365, Chemicon) (1:100 dilutions in complete neurobasal medium) at 12 °C for 1hr. The cell suspension was washed twice with 0.5 % w/v BSA in PBS, centrifuged on a 4.0 % w/v BSA cushion and then incubated with a magnetically labeled goat anti-mouse secondary IgG antibody (20 µl in 1ml complete neurobasal medium) (MAC'S IgG microbeads) at 12°C for 30min. The cell suspension was washed twice with 0.5 % w/v BSA in PBS and centrifuged on a 4.0 % w/v BSA cushion. The cell pellets were suspended in 500ul of 0.5 % w/v BSA in PBS and run through the magnetic set-up to separate p75 ^{NTR} positive neuron (motoneurons). Motoneurons bound to the anti-p75^{NTR} antibody were therefore separated by passing the cell suspension through a magnetized column (Miltenyi Biotech, USA). On passing the cell suspension through the magnetic column, unbound cells are eluted first, and after 2 washing steps with 0.5 % w/v BSA in PBS the column was removed from the magnetic field and the motoneurons eluted from the column with 1ml complete neurobasal medium. The set-up to illustrate the purification principle is as shown in Fig 1. The purified motoneurons were seeded on polyornithin(3 µg/ml) and laminin(3 µg/ml) coated dishes at 2000 cells per 96 well dishes for survival and neurite outgrowth determinations, and at 30,000 per 24 well dishes for cAMP determination and immunoblotting studies. The culture medium used

was neurobasal medium (Gibco) [Table 1] supplemented with the B27 supplement (Gibco) [Table 2], L-glutamine (0.5 mM), 2-mercaptoethanol (25 μM), L-Glutamate (25 μM) and where indicated horse serum (2% v/v), hereafter called complete neurobasal medium.

2.2.3 Assessment of culture purity by Immunofluorescence

The purity of the cultures was assessed by immunofluorescence using antibodies to several neuronal markers which are selectively expressed by embryonic spinal motoneurons. These include Islet 1/2 (Ericson et al., 1992; Arce et al., 1999), choline acetyltransferase (ChAT) (Bataille et al., 1998), SMI-32 (Carriedo et al., 1996; Rao and Weiss, 2004) or p75 (Camu and Henderson, 1992). In our cultures over 90% of the cells were motoneurons.

2.2.3.1 $P75^{NTR}$ immunoflourescence

Motoneurons cultured at 2000 cells per well of a 96-well dish for 24 hours were first washed with 0.5% Tween-20 in Tris-buffered saline (T-TBS, 50mM, pH 7.4), fixed in 4% paraformaldehyde for 20 min at 0°C and blocked in a blocking buffer (4% normal goat serum and 2% bovine serum albumin (BSA) in 0.2% T-TBS) for 1 hr at room temperature. The cells were then incubated with rabbit anti-75^{NTR} antibody (N3908, Sigma) in blocking buffer overnight at 4°C. After washing with 0.2% T-TBS the cells were incubated with fluorescent labeled goat anti-rabbit IgG (Alexa flour 594, Molecular Probes, Oregon, USA) secondary antibody at room temperature for 1hr. The cells were washed and treated with Hoechst (33258) (500ng/ml, Sigma) staining for 15min (to stain blue the nuclei of all viable cells) after which 50μl of mounting medium consisting of

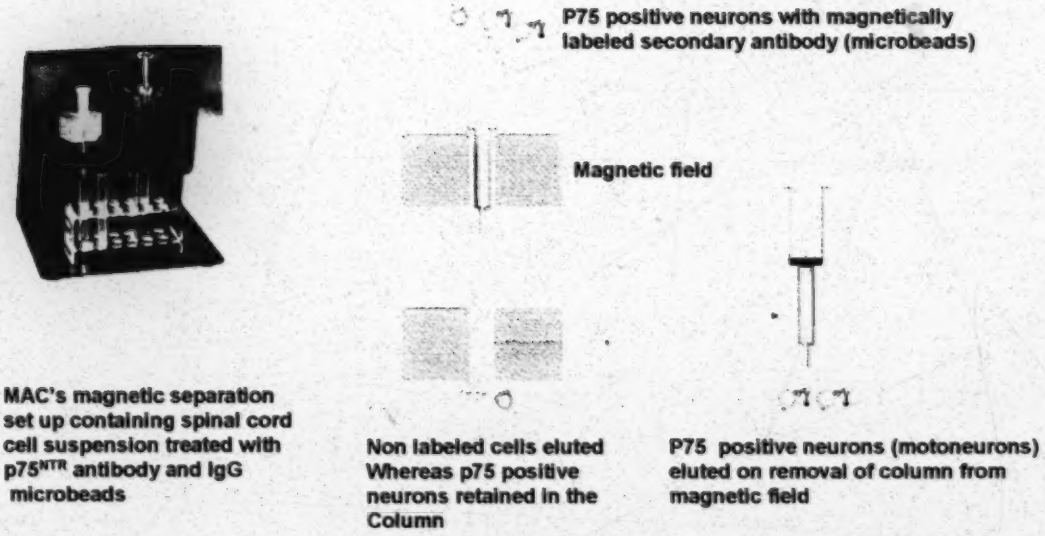
P75 positive neurons with magnetically labeled secondary antibody (microbeads)
Magnetic field
MAC's magnetic separation set up containing spinal cord cell suspension treated with p75NTR antibody and IgG microbeads
Non labeled cells eluted Whereas p75 positive neurons retained in the Column
P75 positive neurons (motoneurons) eluted on removal of column from magnetic field

Figure 1. Set up and principles for immunopurification of motoneurons
Dissociated large cells from the spinal cord collected from Optiprep density centrifugation process were first incubated with mouse anti-$p75^{NTR}$ antibody (MAB 365, Chemicon) at 12°C for 1hr. After series of washing steps the cells were incubated with the secondary antibody (magnetically labeled goat anti-mouse IgG microbeads (MAC's) and incubated for 30min at 12°C. The cell suspension after series of washing was run through a magnetized column in which the magnetically labeled cells (motoneurons) were retained in the column and other cells eluted. The column containing the retained $p75^{NTR}$ positive cells was then removed and the cells eluted as the motoneurons.

Table 1. Composition of Neurobasal medium (Brewer et al., 1993)

	mg/L	μM
Inorganic salts		
$CaCl_2$(anhydrous)	200	1800
$Fe(NO_3)_3.9H_2O$	0.1	0.2
KCl	400	5360
$MgCl_2$(anhydrous)	77.3	812
NaCl	3000	51300
$NaHCO_3$	2200	26000
$NaH_2PO_4.H_2O$	125	900
Other components		
D-glucose	4500	25000
Phenol red	8.1	23
HEPES	2600	10000
Sodium pyruvate	25	230
Amino acids		
L-alanine	2.0	20
L-arginine.HCl	84	400
L-asparagine.H_2O	0.83	5
L-cysteine	1.21	10
L-glutamine	73.5	500
Glycine	30	400
L-histidine.HCl.H_2O	42	200
L-isoleucine	105	800
L-leucine	105	800
L-lysine.HCl	146	5
L-methionine	30	200
L-phenylalanine	66	400
L-proline	7.76	67
L-serine	42	400
L-threonine	96	800
L-tryptophan	16	80
L-tyrosine	72	400
L-valine	94	800
Vitamins		
D-Ca pantothenate	4	8
Choline chloride	4	28
Folic acid	4	8
i-Inositol	7.2	40
Niacinamide	4	30
Pyridoxal.HCl	4	20
Riboflavin	0.4	1
Thiamine.HCl	4	10
Vitamin B12	0.34	0.2

Table 2. Composition of B27 Medium supplement for neurons (Brewer et al., 1993)

Biotin
L-carnitine
Corticosterone
Ethanolamine
D(+)- galactose
Glutathione (reduced)
Linoleic acid
Linolenic acid
Progesterone
Putrescine
Retinyl acetate
Selenium
T3(triodo-l-thyronine)
DL-alfa tocopherol(vitamin E)
DL-alfa tocopherol acetate
Proteins
Albumin,bovine
Catalase
Insulin
Superoxide dismutase
Transferrin

0.005% N-propylgallate and 72% glycerol in 1x TBS was added to cover the cells for fluorescence microscopic observation. The motoneurons were observed and images captured under fluorescence microscope using the Nikon TE300 inverted microscope equipped with the Nikon digital camera DXM-1200 (Nikon Canada) and $p75^{NTR}$ immunopositive neurons used as an assessment of the purity of the motoneuron cultures. Representative photomicrographs of p75NTR immunopositive motoneurons are shown in Fig 2.

2.2.4 Assessment of motoneuronal survival

Survival of cultured motoneurons was determined by measuring the reduction of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]. In this method, during 1 hour incubation, living neurons convert the MTT tetrazolium component of the dye solution into a blue formazan product (Berridge and Tan, 1993). The live motoneurons were identified under bright field microscopy as dark-blue cells and the percentage of the live motoneurons was determined. Representative photomicrographs of neurons treated and untreated with MTT reagent are shown in Fig 3.

2.2.5 Motoneuron treatments

Appropriate agents were applied to modulate intracellular cAMP levels. To elevate cAMP we used the membrane permeable analogue of cAMP, dibutyryl cAMP (DbcAMP, 1mM) (Calbiochem) and forskolin (3 -15μM) (Sigma), which activates adenylyl cyclase to convert adenosine triphosphate (ATP) to cAMP. In order to prevent the metabolism of cAMP, 1-Isobutyl-3-methylxanthine (IBMX) (50μM) (Calbiochem) a

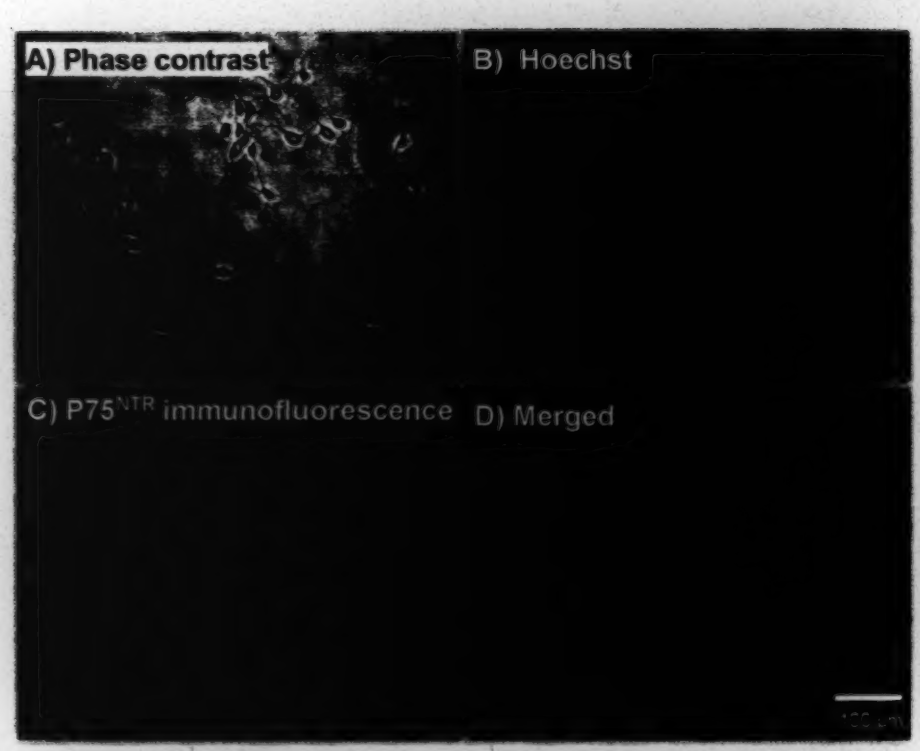

Figure 2. P75^{NTR} immunofluorescence of motoneurons
Motoneurons cultured for 24 hr in a 96 well dish were incubated with rabbit anti- p75^{NTR} antibody overnight at 4°C. After series of washing steps the neurons were incubated with goat anti-rabbit Alexa fluor 594 IgG secondary antibodies followed by Hoechst staining at room temperature for 1hr and 15min respectively. The cells were mounted in a mounting medium and observed under fluorescence microscope. The select photomicrographs represent the phase contrast (A), Hoechst positive (B), p75^{NTR} positive (motoneurons) (C) and Hoechst and p75^{NTR} positive merged observed.

Figure 3 Representative photomicrographs of motoneurons before and after treatment with MTT reagent. A) MTT untreated cultures showed neurons (grey arrowed) with uniform colorations. **B)** MTT treated cultures showed viable neurons stained dark-blue (shown dark) (black arrowed) with MTT reagent whereas non-viable neurons are unstained (white arrowed)

general phosphodiesterase inhibitor and a phosphodiesterase type 4 inhibitor Rolipram (10μM) (AG Scientific) were used. To reduce intracellular cAMP levels, SQ22536 (300-500 μM) (Calbiochem), a soluble adenylate cyclase inhibitor that prevents conversion of ATP to cAMP, was applied. H89 (1-20μM) (Calbiochem) a specific PKA inhibitor and the cAMP antagonist Rp-cAMP (200-500μM) (Calbiochem) that inhibits PKA at cAMP binding site on PKA, were also used to inhibit PKA action. The various pharmacologic agents used and their targets in the cAMP generation as well as their proposed effects on neurite outgrowth are illustrated in Fig 4. To investigate Erk activation by immunoblotting, BDNF (50ng/ml), MAP kinase kinase inhibitor PD98059 (50μM) and the tyrosine kinase inhibitor K252a (500nM) were used as controls. The doses indicated for the various agents above were based on concentrations used *in vitro* in other neuronal cells and on the dose responses obtained in motoneurons for these agents. Unless otherwise stated motoneuron treatments started at the time of plating and continued for up to 24h.

2.2.6 Assessment of neuronal viability under selected treatment conditions

To establish the viability of the motoneurons under the conditions of the treatment with the various pharmacologic agents, Hoechst viability assay was done. Motoneurons were treated with Forskolin (3 μM), DbcAMP (1 μM), IBMX (50 μM), Rolipram (10 μM), RpcAMP (500 μM), H89 (20 μM) and SQ22356 (500 μM) for 24hrs, and with Hoechst staining reagent (500ng/ml) for 15min in 0.2% T-TBS. The extent of cell viability was assessed by determining the percent of neurons that stained blue with Hoechst and show no nuclei fragmentation.

2.2.7 Parameters of neurite outgrowth

The number of neurons with neurites and the length of the longest neurites were determined from images captured using the Nikon TE300 inverted microscope equipped with the Nikon digital camera DXM-1200 (Nikon Canada). The images were analyzed using Northern Eclipse Elite V6.0 image capture and analysis software (Empix Imaging, Canada). The motoneurons with processes >10 μm were counted as neurons with neurites from 10 randomly selected fields per well performed in triplicate (a total of 9 wells) and the percent of neurons with neurites was calculated. Neurite length was determined by tracing the longest neurite from the cell body of the neuron and following the entire length of the neurite in 100-150 neurons per well per experiment (neurites of more than 300 neurons were measured per per treatment). In all cases the experiments were done in triplicate and repeated three times (n = 9, total of number of wells).

2.2.8 Measurement of cAMP in cultured motoneurons

Quantification of cAMP in cultured motoneurons was carried out by competitive enzyme immunoassay using a commercially available ELISA kit (R&D Systems). After treatment of the motoneurons the culture media were aspirated and 350μl of 0.1N HCl added and incubated at 37°C for 10 min to lyse the neurons and prevent degradation of cAMP by phosphodiesterases. The resulting solution was centrifuged at 15,000 rpm for 5 min. The supernatant was used for the cAMP determination. The pellets were dissolved in Radio-Immunoprecipitation Assay (RIPA) buffer [Table 3] and the protein determined using the Bicinchoninic Acid BCA kit (Pearce, USA). The amount of cAMP was expressed as pmol/μg of protein.

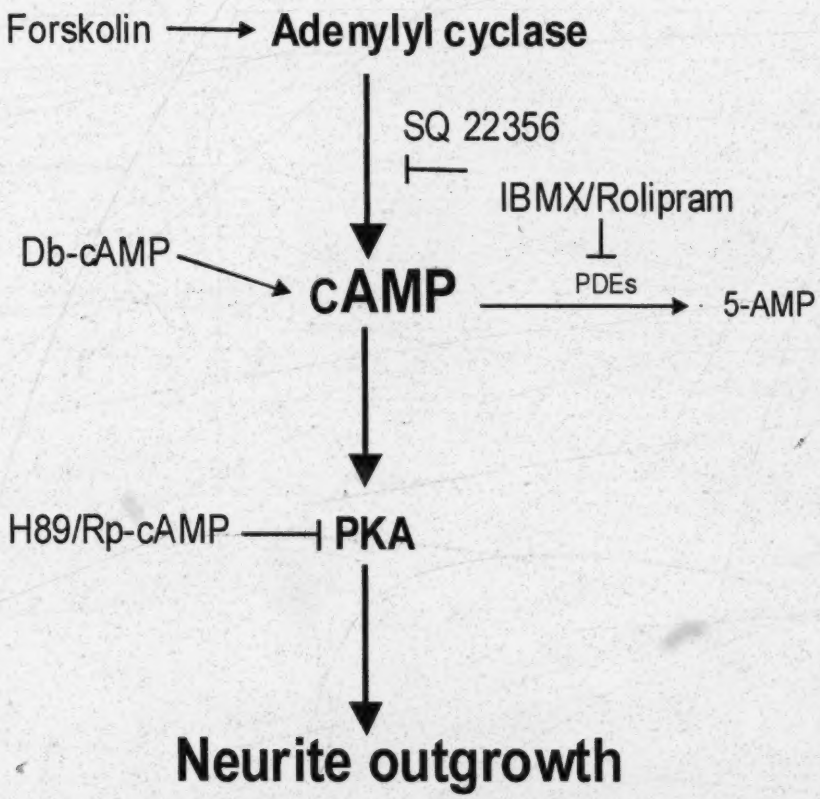

Figure 4. Targets for pharmacological investigation of cAMP effects on neurite outgrowth in motoneurons. Forskolin was used to stimulate adenylyl cyclase to generation of cAMP, whereas SQ22356 was to inhibit adenylyl cyclase to reduce intracellular cAMP. Intracellular cAMP could be raised through the use of the cell permeable analog DbcAMP and/or inhibition of cAMP phosphodiesterases using 3-Isobutyl-1-methyl xanthine (IBMX) and Rolipram. Cyclic AMP generally mediates its effects through PKA. Blocking PKA using the PKA inhibitors H89 and RpcAMP would help to establish if PKA is involved in neurite outgrowth in motoneurons.

Table 3. Composition of RIPA buffer

Composition	Concentration
Tris HCl	50 mM
NaCl	150 mM
Nonidet P40	1%
Sodium deoxycholate	0.5%
EDTA	2 mM
$MgCl_2$	2 mM
Correct pH to 7.1	

2.2.9 Erk immunoblotting

After experimental treatment motoneurons were washed with ice-cold PBS containing 1 mM sodium orthovanadate and 10mM sodium fluoride and harvested in RIPA buffer. An aliquot was taken for protein determination. Buffer containing 40mM Tris–HCl pH 6.8, 1% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue was added to the sample and boiled for 2 min. Equal amount of proteins (20μg) were separated by SDS-PAGE on 10% polyacrylamide gels containing 0.1% SDS and transferred overnight at 4 °C to PVDF membranes in 25 mM Tris: 192 mM glycine, 16% methanol buffer, pH 8.3, containing 0.1% SDS. Membranes were blocked for 1 h in TBS, 0.1% Tween-20 (TTBS) containing 5% non-fat milk (blocking buffer) and incubated overnight in the primary antibody solution prepared in TTBS containing 5% bovine serum albumin. The primary antibodies used were anti-phospho-ERK1/2 (1:1000) and anti-total ERK 1/2 (1:1000) from Cell Signaling Technology (Beverly, MA, USA). Membranes were washed 20 minutes with TTBS, 20 minutes with TBS and then 20min with TTBS and incubated for 1h with the secondary antibody goat anti-rabbit horseradish-peroxidase conjugated Immunoglobulin G (Pierce, US) (1:2000) in blocking buffer (0.1% Tween-20, 5% non-fat milk in Tris buffered saline) at room temperature with gentle agitation. In all cases the levels of total-ERK was used as a loading control. Immunoreactivity was detected by Enhanced Chemo Luminescence (ECL-Plus, Amersham Biosciences).

2.2.10 Statistics

Where indicated the data were analyzed using the Graphpad Instat software (US). The statistical significance was determined where indicated using the Student's t-test and One-Way anaylsis of variance using the Student Newman Keuls test and significance accepted at $p < 0.05$. The data are presented as the mean $\pm$ standard error (SEM).

2.3 RESULTS

2.3.1 Optimum conditions established for motoneuron growth and survival

High levels of intracellular cAMP in neonatal DRG neurons 1d but not 4d postnatal in rats were sufficient to promote neurite outgrowth on the inhibitory central myelin/MAG substrate (Cai et al., 2001). We investigated whether 1) the levels of intracellular cAMP in embryonic motoneurons are sufficient to promote neurite outgrowth on a permissive substrate and 2) elevation or depression of these levels promotes and reduces axon outgrowth as predicted

We isolated and cultured motoneurons from rat embryos at day 15 and established the conditions for survival of these neurons prior to manipulating intracellular levels of cAMP to study the role of cAMP in neurite outgrowth on a permissive substrate. It was important to examine whether motoneurons survive in the absence of exogenously added neurotrophic factors since it has been documented that the neurotrophins induce elevation of cAMP in some neuronal types (Cai et al., 1999). We observed that motoneurons cultured in the presence or absence of exogenous growth factors did not show any morphological differences, and extended neurites normally (Fig. 5A, B). Over a 12d

period, the presence or absence of exogenous growth factors (BDNF, GDNF and CNTF) did not affect neuron survival: there was no difference in survival of the motoneurons whether or not these factors were present in the culture medium (Fig. 5C).

2.3.2 Intracellular cAMP is required for neurite outgrowth in motoneurons

Physiologically cAMP is produced from ATP when adenylyl cyclase is activated by the G_s subunit of heterotrimeric G-proteins (Houslay and Kolch, 2000). We used the readily soluble and potent adenylyl cyclase inhibitor SQ22356 (Harris et al., 1979) in order to reduce intracellular cAMP levels and to ask whether reduction in cAMP inhibits neurite outgrowth in cultured motoneurons. We first established the effective concentrations of SQ22356 in reducing intracellular cAMP in motoneurons at 2 and 18hr of treatment. We used SQ22356 at concentrations of 300-500μM that inhibit neuronal proliferation and neurite outgrowth in cerebellar neurons and PC12 cells (Gysbers and Rathbone, 1996; Gomes et al., 1999). SQ22356 was effective in rapidly reducing intracellular cAMP levels in a concentration-dependent manner (Fig. 6A). With decreasing levels of intracellular cAMP the percent of motoneurons that extended neurites dramatically declined (Fig. 6B) and the length of neurite extension progressively fell (Fig. 6C). The greatest reductions in the percentage of motoneurons that extended neurites and in neurite outgrowth were observed at 500μM of SQ22356. At this inhibitor concentration the levels of intracellular cAMP were reduced by 50 to 60% and only 5% of motoneurons extended neurites. Representative phase contrast photomicrographs in Fig 6 D, E illustrates the effects of SQ22356 (500μM) on neurite

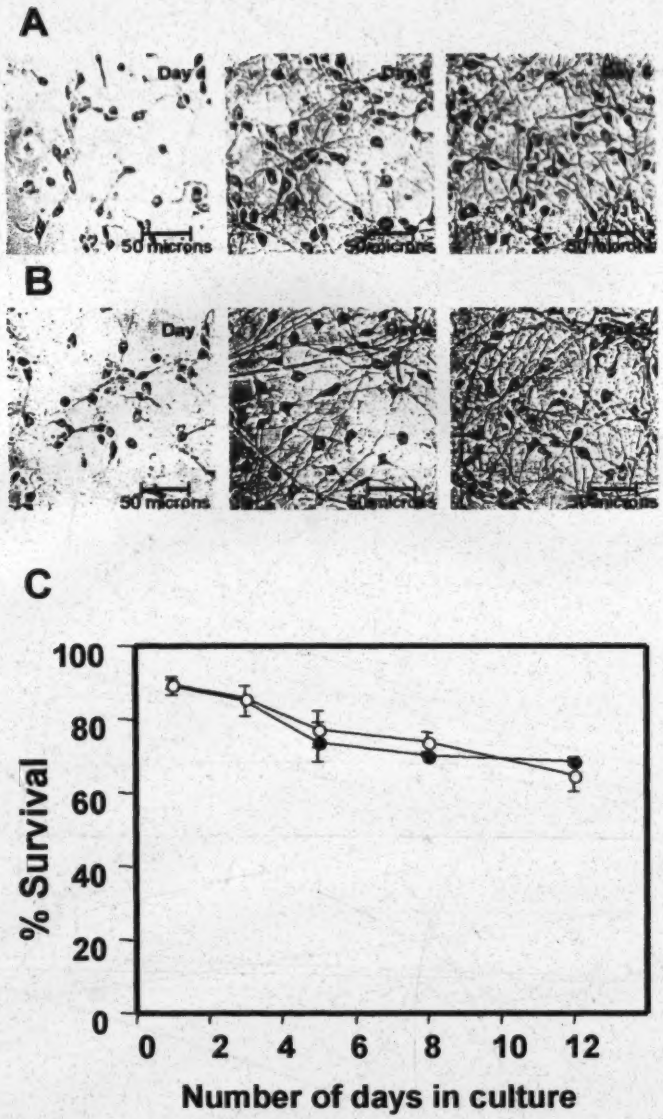

Figure 5. Survival of motoneurons in the presence or absence of exogenous neurotrophic factors. Motoneurons were cultured at a density of 2000 cells per 96-well dish for up to 12 days in: **(A)** complete neurobasal medium without added growth factors and **(B)** complete neurobasal medium containing BDNF (10 ng/ml), CTNF (10 ng/ml) and GDNF (10 ng/ml). Representative phase contrast micrographs of motoneuron cultures taken on selected days are shown. **(C)** Percentage of survival of motoneurons cultured in (•) complete neurobasal medium alone and (o) complete neurobasal medium containing BDNF (10 ng/ml), CNTF (10 ng/ml) and GDNF (10 ng/ml) determined by the MTT assay. Data are expressed as mean ± SEM ($n = 4$).

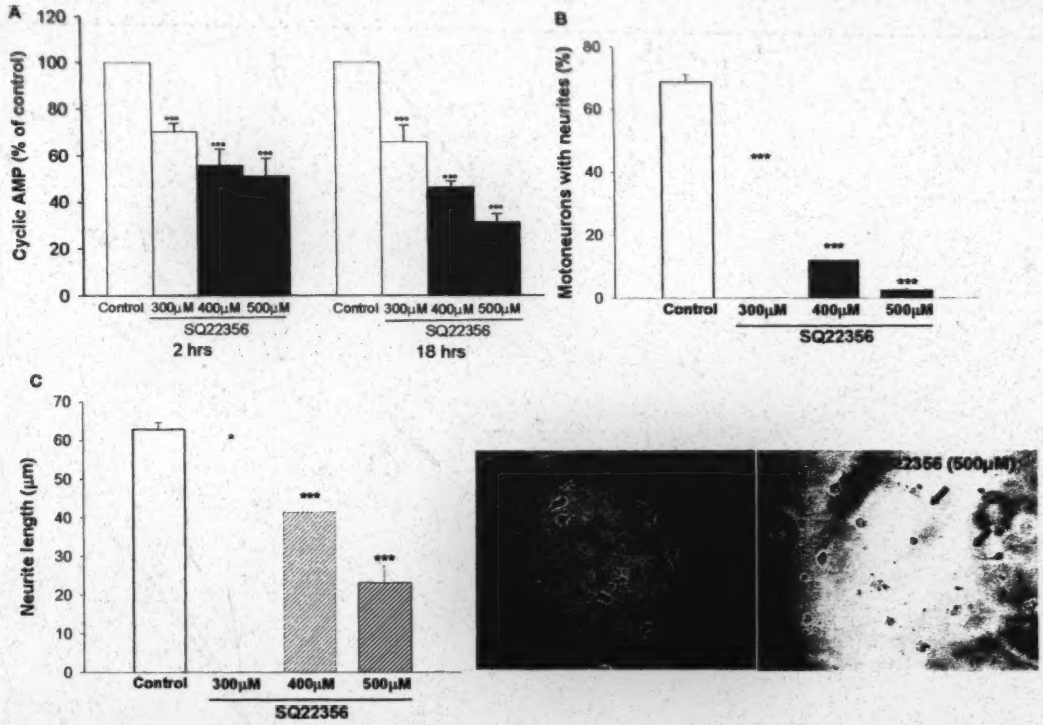

Figure 6. SQ22356 reduces intracellular cAMP levels and inhibits neurite outgrowth. Motoneurons were cultured at a density of 2000 cells per 96-well dish or 30,000 per 24-well dish and treated with SQ22356 (300 - 500μM) at the time of plating. **(A)** Intracellular cAMP was determined after 2 and 18h. The percent of motoneurons which extended neurites **(B)** and the neurite length **(C)** were determined 18h after treatment. The data represent the Mean ± SEM. The experiment was performed in triplicates and repeated three times. The data was analyzed by the Student's t-test. Significance is indicated with respect to the untreated control * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$. Phase contrast micrographs of (D) untreated and (E) SQ22356 (500μM) treated neurons illustrate the distinction in neurons with neurites and neurons with longer neurites.

outgrowth. The correlation between reduced levels of intracellular cAMP, percentage of motoneurons with neurites and their neurite length, strongly indicate that intrinsic intracellular cAMP is important for axon outgrowth in motoneurons. At this concentration of 500 μM over 95% of the neurons were viable as demonstrated by Hoechst staining viability assay (Fig 7). However concentrations above 500μM were neurotoxic, resulting in obvious morphological changes characteristic of cell death.

2.3.3 Elevation of cAMP enhances outgrowth of neurites in motoneurons

The question now arises as to whether pharmacological elevation of cAMP result in increased neurite outgrowth from motoneurons as predicted from reduced outgrowth by SQ22356 treatment. We first investigated the effect of exogenous dibutyryl cAMP (DbcAMP) a readily soluble analog of cAMP on neurite outgrowth and extension. DbcAMP at a concentration of 1mM induced neurite outgrowth in Neuro2A cells (Mulder et al., 2004) and increased neurite length in NG108-15 cells (Tojima et al., 2003), and in cerebellar and DRG neurons cultured on purified CNS myelin (Cai et al., 1999). We treated the motoneurons with dbcAMP at 1mM at the time of cell plating. The number of motoneurons that extended neurites (Fig 8A) and the mean length of the longest neurites (Fig 8 B, C, D) were significantly increased in medium containing 1mM of DbcAMP. At this concentration of 1mM over 95% of the neurons were viable as demonstrated by Hoechst staining viability assay (Fig 9).

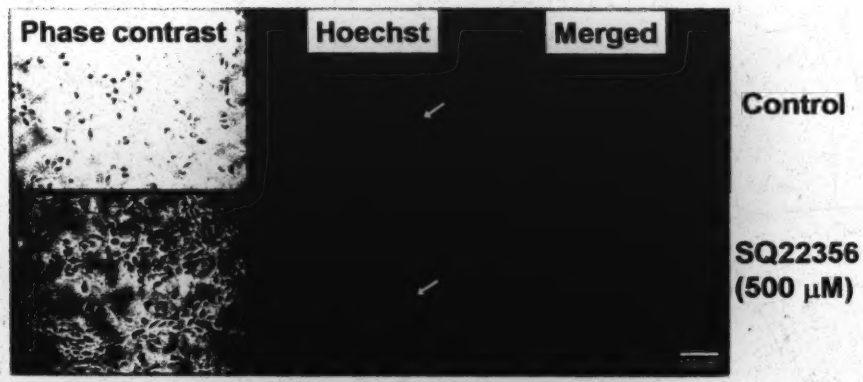

Figure 7. Hoechst viability assay on SQ22356 treated neurons
Motoneurons were cultured in 96 well dishes and treated with SQ22356 (500μM) and cultured for 24hrs. The neurons were washed and treated with Hoechst (33258) 500ng/ml for 15 min, mounted in mounting medium and observed for apoptosis indicated by fragmented nuclei. The photomicrographs show the first panel from left as a phase contrast, second panel as Hoechst stained neurons and the last as the merged images of both pictures. Arrowed neurons show non viable apoptotic neurons with fragmented nuclei.

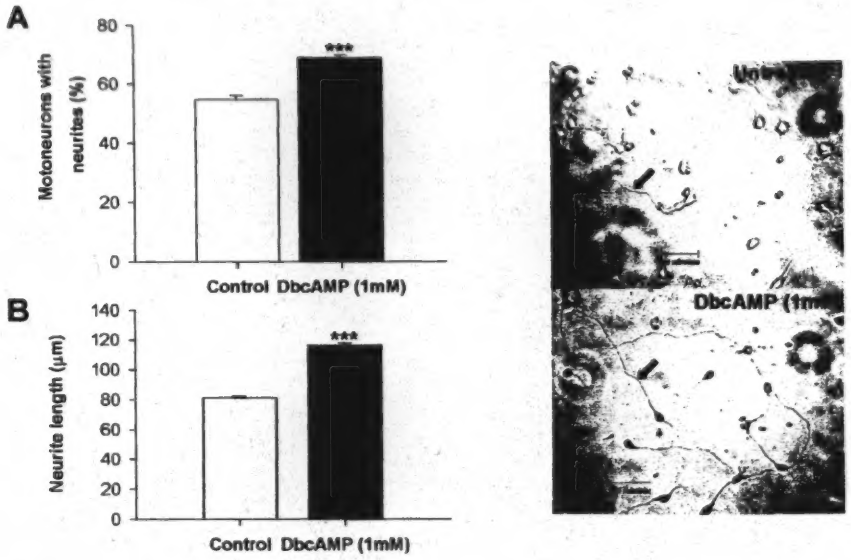

Figure 8. Effects of DbcAMP on neurite outgrowth

Motoneurons were plated at 2000 cells per well of a 96 – well dish and treated with 1mM of DbcAMP at the time of seeding. **(A)** The percent of neurons with neurites and **(B)** the mean length of the longest neurites were determined after 24 h in culture. The experiment was done in triplicate and repeated three times. The differences between treated and untreated controls were determined by the Student t-test. The results are presented as Mean ± SEM, n = 9, *** $p < 0.001$. Representative phase contrast micrographs of untreated motoneurons (C) showing shorter neurite (arrowed) as compared with DbcAMP (1 mM) treated motoneurons (D) with longer neurite (arrowed)

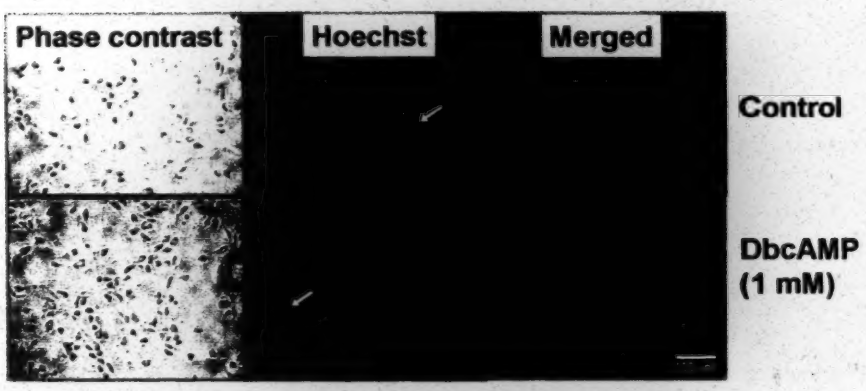

Figure 9. Hoechst viability assay in DbcAMP treated neurons
Motoneurons were cultured in 96 well dishes and treated with DbcAMP (1mM) for 24hrs. The neurons were washed and treated with Hoechst (33258) 500ng/ml for 15 min, mounted in mounting medium and observed for apoptosis indicated by fragmented nuclei. The photomicrographs show the first panel from left as a phase contrast, second panel as Hoechst stained neurons and the last as the merged images of both pictures. Arrowed neurons show non viable apoptotic neurons with fragmented nuclei.

2.3.4 Forskolin increased intracellular cAMP and promoted neurite outgrowth

Next we increased the endogenous levels of cAMP pharmacologically. Previous work showed that Forskolin, which stimulates adenylyl cyclase, effectively increased axon outgrowth in PC12 cells (Hansen et al., 2003) and DRG neurons (Neumann et al., 2002). Raised levels of cAMP by forskolin in concentrations ranging from 3 to 10μM also increased survival and neurite outgrowth in several different groups of neurons *in vitro* (Hansen et al., 2003; Peng et al., 2003; Ivins et al., 2004). We found that at these concentrations, there was an early dose-dependent increase in cAMP in cultured motoneurons (Fig. 10A). Treatments longer than 2h with forskolin at concentrations of 7μM or higher resulted in motoneuronal death. To assure the health of the neurons, we used 3μM forskolin for further experiments. The increased in intracellular cAMP elicited by 3μM forskolin resulted in significantly increased numbers of motoneurons that extended neurites at 24h (Fig. 10B) and increased mean length of the longest neurites after 12 and 24h (Fig. 10C). In the untreated motoneurons the percent of motoneurons that extended neurites remained unchanged between 12 and 24h; however the percentage increased significantly in forskolin-treated neurons. This observation suggests that between 12 and 24h more motoneurons are induced to extend neurites in response to forskolin-induced elevation of cAMP. At the forskolin concentration of 3 μM over 95% of the neurons were viable as demonstrated by Hoechst staining viability assay (Fig 11).

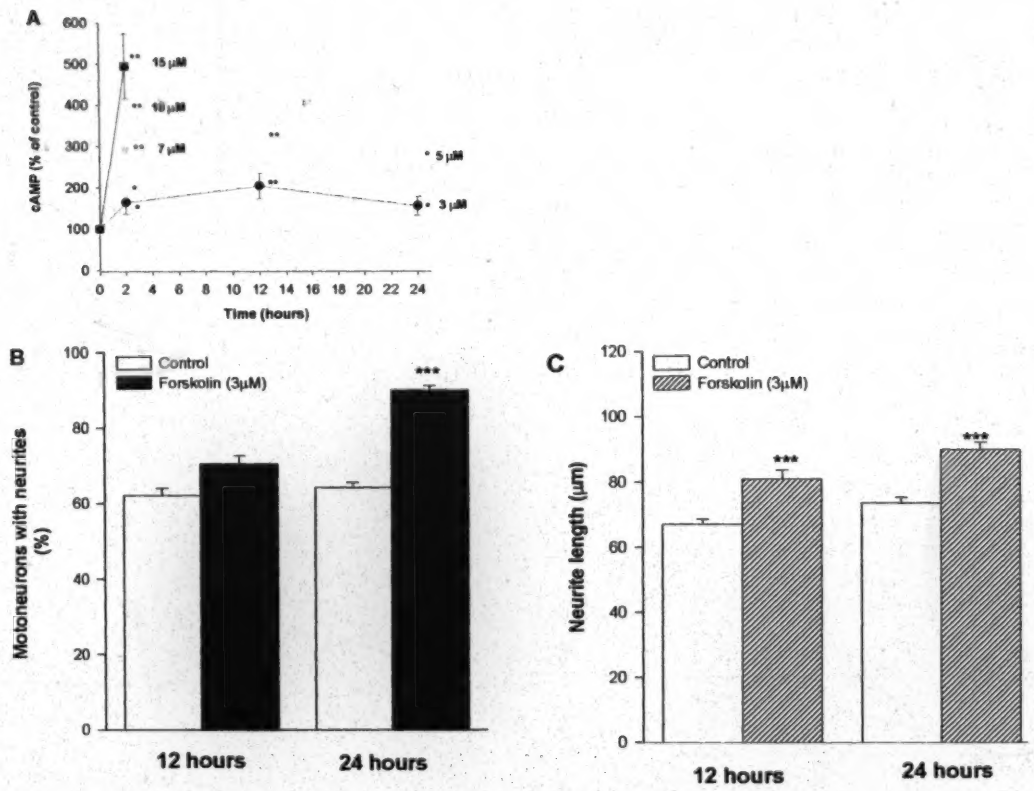

Figure 10. Forskolin increases cAMP and promotes neurite outgrowth. Motoneurons were cultured at a density of 2000 cells per 96-well dish or 30,000 per 24-well dish and treated with various concentrations of forskolin at the time of plating. **(A)** Intracellular cAMP levels were determined at selected time points. The experiment was discontinued with forskolin concentrations over 5 µM since the majority of the neurons were dead at 12 hours **(B)** The percent of motoneurons that extend neurites and **(C)** the mean length of the longest neurites after 12 and 24 h in culture respectively are shown. The data represent the Mean ± SEM, n = 9. Significance is indicated with respect to the untreated control * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$

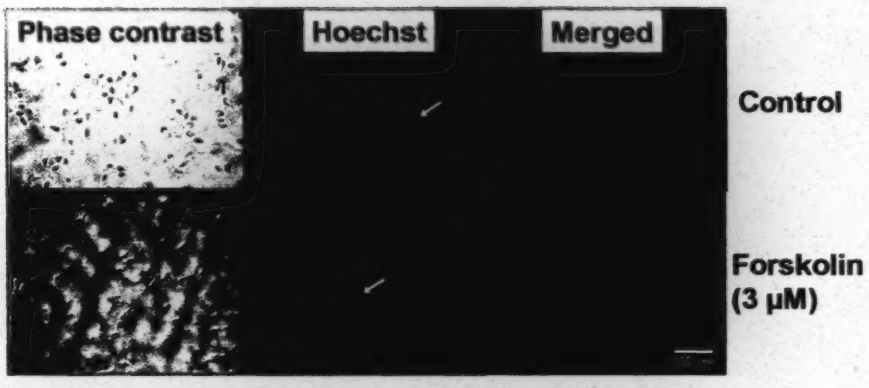

Figure 11. Hoechst viability assay in forskolin treated neurons.
Motoneurons were cultured in 96 well dishes and treated with forskolin (3 μM) for 24hrs. The neurons were washed and treated with Hoechst (33258) 500ng/ml for 15 min, mounted in mounting medium and observed for apoptosis indicated by fragmented nuclei. The photomicrographs show the first panel from left as a phase contrast, second panel as Hoechst stained neurons and the last as the merged images of both pictures. Arrowed neurons show non viable apoptotic neurons with fragmented nuclei.

2.3.5 Phosphodiesterase inhibitors Rolipram and IBMX raised intracellular cAMP and promoted neurite outgrowth

An alternative way to increase intracellular cAMP is to inhibit its degradation. Under physiological conditions cAMP is quickly degraded by the cAMP phosphodiesterases (Houslay, 1998; Houslay and Adams, 2003). Therefore we used the general phosphodiesterase inhibitor isobutyl-3-methyl xanthine (IBMX) and the type 4 phosphodiesterase inhibitor rolipram. Rolipram is the archetypal inhibitor of the type 4 phosphodiesterases (Houslay and Kolch, 2000) which are the most abundant group of the phosphodiesterases in neuronal tissues (Jin et al., 1999). Rolipram has been used *in vitro* at concentrations ranging from 0.1 to 100μM which were effective in increasing cAMP levels and removing MAG inhibition to promote neurite outgrowth on a non-permissive substrate (Houslay and Kolch, 2000; Gao et al., 2003; Nikulina et al., 2004a). Similarly, IBMX has been used at concentrations ranging between 50 and 100μM to inhibit cAMP phosphodiesterases to promote survival of motoneurons and immortalized neuronal cells in culture (Hansen et al., 2003; Peng et al., 2003; Araki et al., 2005). The effect of the cAMP phosphodiesterases on neurite outgrowth has not been examined in motoneurons.

Cultured motoneurons were treated with Rolipram (10μM) or IBMX (50μM) at the time of plating and cAMP was measured after 2, 12 and 24h in culture. We found that both inhibitors were effective in increasing intracellular cAMP when compared with untreated controls (Fig. 12A). The increased levels of intracellular cAMP by IBMX and Rolipram were associated with significantly increased number of motoneurons that extended neurites (Fig. 12B) and increased mean length of the longest neurites (Fig. 12C) at 12 and 24h. At Rolipram concentration of 10 μM and IBMX concentration of 50 μM

over 95% of the neurons were viable as demonstrated by Hoechst staining viability assay (Fig 13).

2.3.6 Activation of PKA by cAMP is required for outgrowth of neurites in motoneurons

The most common intracellular target of cAMP is PKA. PKA is responsible for many of the actions attributed to cAMP (Houslay and Kolch, 2000) including neurite outgrowth in many cells such as PC12 cells and DRG neurons (Kao et al., 2002; Qiu et al., 2002b; Qiu et al., 2002a). Nonetheless there are other systems in which cAMP-induced neurite outgrowth is independent of PKA activation (Charles et al., 2003a). We therefore tested if PKA activation is required for neurite extension in cultured motoneurons. The selective PKA inhibitor H89 (Chijiwa et al., 1990) effectively inhibits neurite outgrowth on permissive and non-permissive substrates at concentrations ranging from 400nM to 25μM (Qiu et al., 2002b; Lara et al., 2003). We found that in motoneurons H89 caused a substantial and significant decrease in neurite length at concentrations between 10μM and 20μM after 24h (Fig 14A). This result indicates that intrinsic intracellular cAMP-regulated neurite outgrowth in motoneurons like the DRG sensory neurons are mediated through PKA.

When basal intracellular cAMP levels was elevated by treatment with forskolin (3μM) or dbcAMP (1mM), the neurite outgrowth of motoneurons was blocked by co-administration of H89 (Fig 14B,C). The same result was obtained with another PKA inhibitor RpcAMP (500 μM) that was tested in the absence or presence of 3μM forskolin (Fig. 14D). These experiments show that the increase in neurite outgrowth in

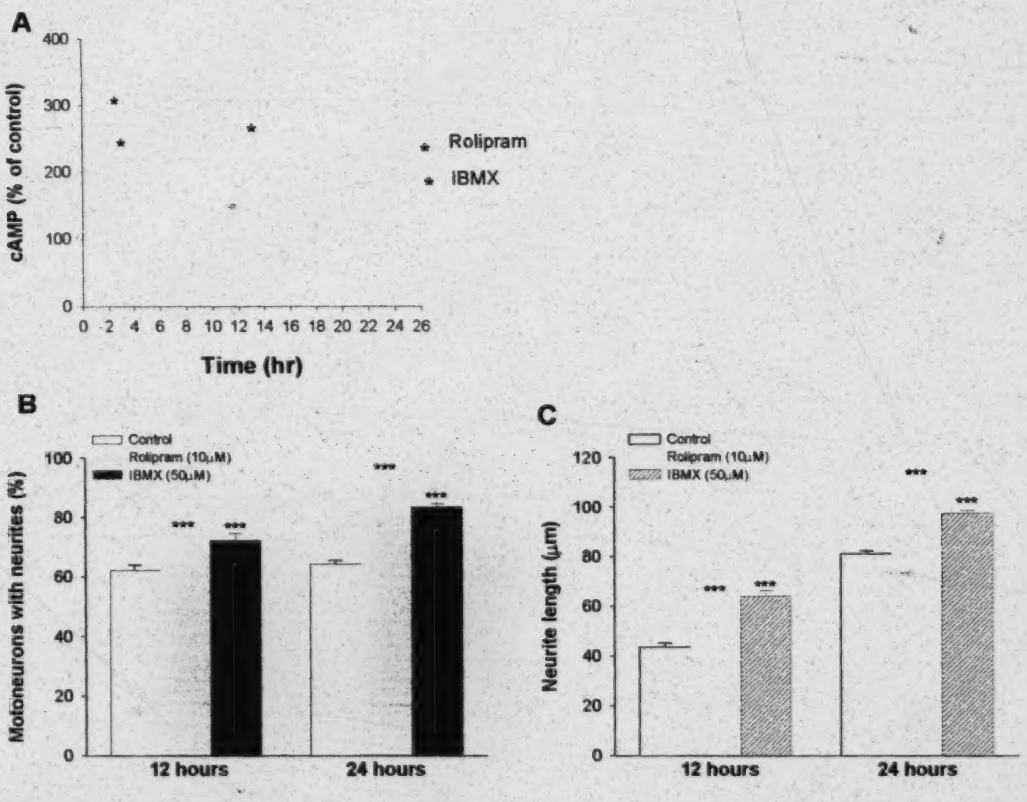

Figure 12 Inhibition of phosphodiesterases raises intracellular cAMP and promotes neurite outgrowth.

Motoneurons cultured at a density of 2000 cells per 96-well dish or 30,000 per 24-well dish were treated with Rolipram (10 μM) or IBMX (50 μM) at the time of plating. **(A)** cAMP was measured at 2 hr, 12h and 24h. **(B)** Percent of motoneurons that extended neurites **(C)** and the mean length of longest neurites over the 24h period. The data represent the Mean ± SEM, n = 9. Data was analyzed by the Student's t-test. Significance is indicated with respect to the untreated control * $p<0.05$, *** $p < 0.001$.

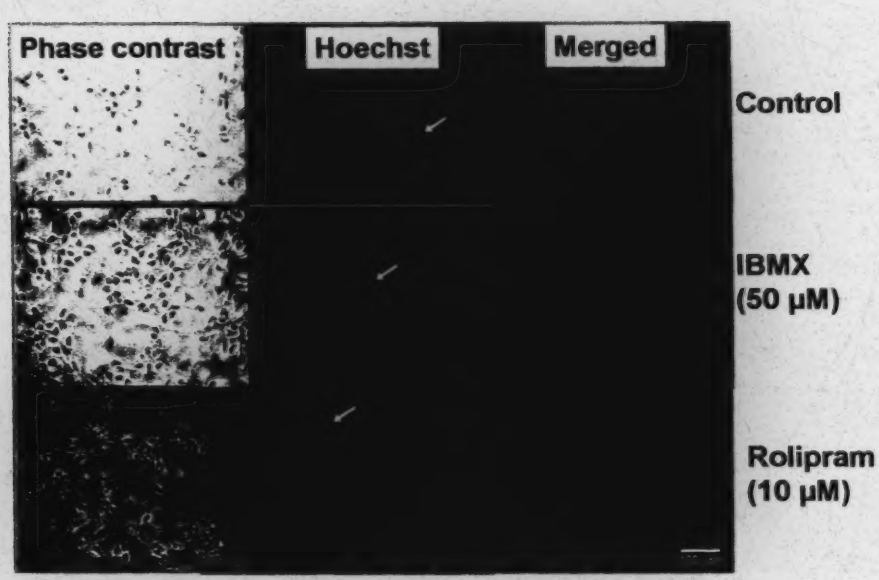

Figure 13. Hoechst viability assay in Rolipram and IBMX treated neurons
Motoneurons were cultured in 96 well dishes and treated with either Rolipram (10µM) or IBMX (50µM) for 24hrs. The neurons were washed and treated with Hoechst (33258) 500ng/ml for 15 min, mounted in mounting medium and observed for apoptosis indicated by fragmented nuclei. The photomicrographs show the first panel from left as a phase contrast, second panel as Hoechst stained neurons and the last as the merged images of both pictures. Arrowed neurons show non viable apoptotic neurons with fragmented nuclei.

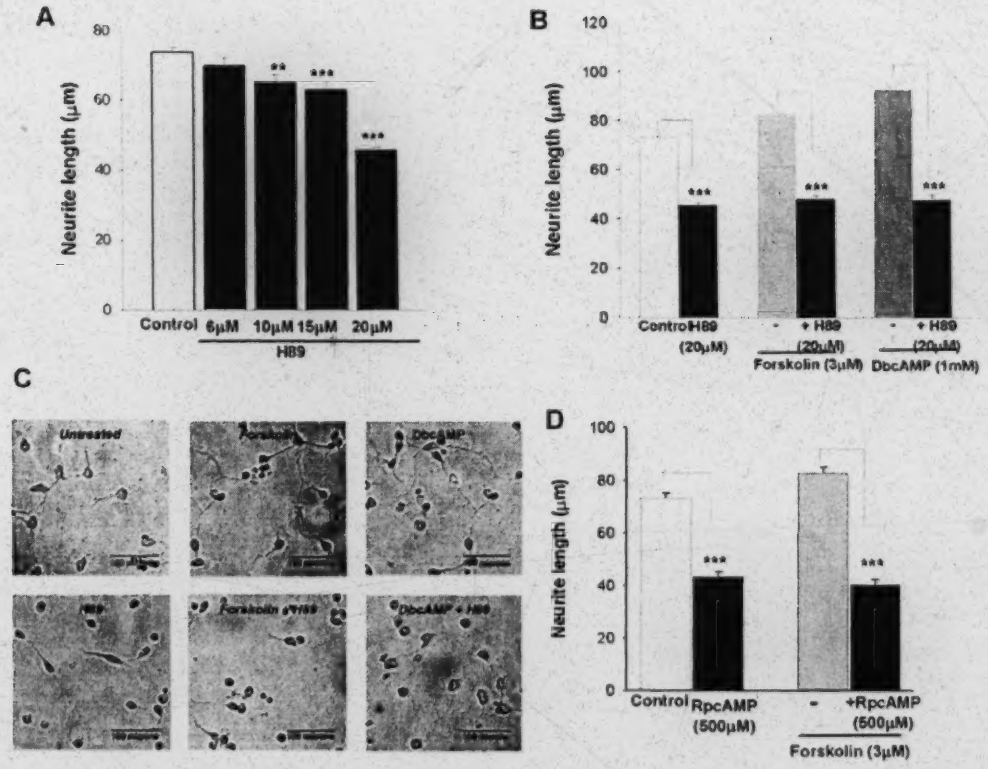

Figure 14. PKA mediates neurite outgrowth in motoneurons
Motoneurons cultured at a density of 2000 cells per 96-well dish were treated at the time of plating with **(A)** H89 at concentrations of 6 to 20µM and neurite length was measured after 24h and **(B)** forskolin (3µM) or dbcAMP (1mM) in the presence or absence of H89 (20µM) and neurite length was measured after 24h. **(C)** Phase-contrast images of motoneurons treated as indicated in (B). **(D)** Motoneurons were treated with RpcAMP (500µM) alone or together with forskolin and the length of neurite lengths measured. The experiments were done in triplicate and repeated three times. Data were analyzed with the Student's t-test and One Way analysis of variance with Newman-Keuls post test. The results are presented as Mean ± SEM, n = 9. Significance is accepted at $p<0.05$, ** $p < 0.01$, *** $p < 0.001$.

motoneurons that follow elevation of cAMP above basal levels is also mediated by PKA. At these inhibitor concentrations of H89 (20μM) and RpcAMP (500 μM) the neurons were viable with no difference observed in viability between treated and untreated controls as illustrated in Fig. 15.

2.3.7 Cyclic AMP does not activate the Ras-Erk pathway in motoneurons

The well established signaling pathway that regulates neurite outgrowth in neurons is the Ras-Erk pathway (Kaplan and Miller, 2000). Previous evidence indicated that elevation of cAMP leads to activation of the Ras-Erk pathway in PC12 cells, hippocampal neurons (Grewal et al., 2000; Kiermayer et al., 2005), DRG neurons (Gao et al., 2003) and many other cell types (Stork and Schmitt, 2002; Chu et al., 2006). Hence we examined Erk activation in motoneurons under conditions in which the levels of cAMP and neurite outgrowth were shown to be effectively modulated in the neurons. Erk activation by phosphorylation (p-Erk) was not affected by application of the PKA inhibitor H89 (20μM) and the adenylyl cyclase inhibitor SQ22356 (500μM) that had effectively reduced cAMP levels and reduced neurite outgrowth in the motoneurons (Fig. 16A). The cAMP analogue, dbcAMP at a concentration of 1mM that enhanced neurite outgrowth (Fig.8) did not lead to Erk phosphorylation. In contrast, the neurons responded robustly to BDNF (50ng/ml) with Erk phosphorylation in the same period of incubation. This process was mediated by tyrosine kinase because the Erk phosphorylation was reduced by the tyrosine kinase inhibitor K252a (500nM) that is normally used to inhibit Trk receptor activation (Hashimoto, 1988; Tapley et al., 1992) (Fig. 16A). Therefore,

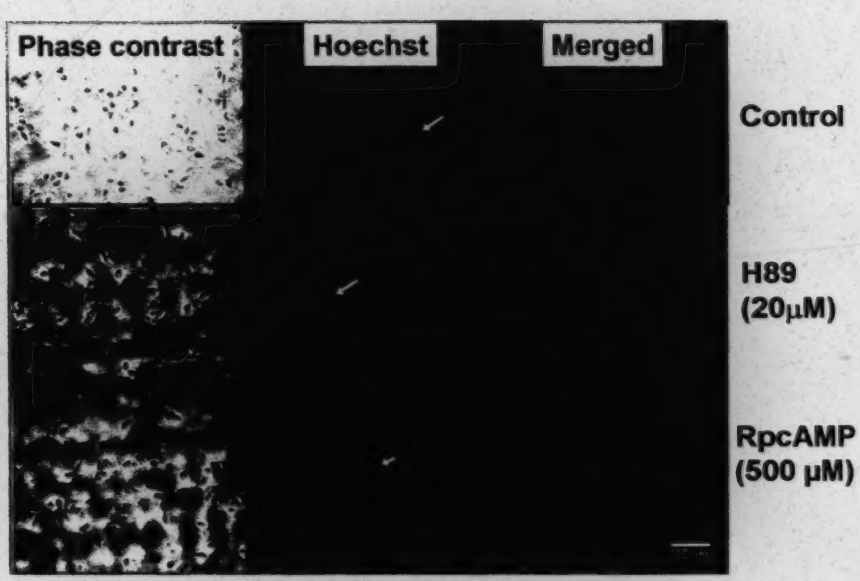

Figure 15. Hoechst viability assay in H89 and RpcAMP treated neurons.
Motoneurons were cultured in 96 well dishes and treated with either RpcAMP (500μM) or H89 (20μM) for 24hrs. The neurons were washed and treated with Hoechst (33258) 500ng/ml for 15 min, mounted in mounting medium and observed for apoptosis indicated by fragmented nuclei. The photomicrographs show the first panel from left as a phase contrast, second panel as Hoechst stained neurons and the last as the merged images of both pictures. Arrowed neurons show non viable apoptotic neurons with fragmented nuclei.

cAMP in motoneurons are unlikely to regulate Erk phosphorylation and hence Erk activation.

The levels of Erk phosphorylation observed under starvation conditions (serum deprivation) were unusually high (Fig. 16A, B). To determine whether this Erk activation was due to BNDF acting via an autocrine mechanism, we examined Erk phosphorylation under conditions of K252a-mediated blockade of the motoneuronal TrkB receptors. At a concentration of 500nM, K252a only partially reduced Erk activation in BDNF-treated as well as in untreated neurons (Fig. 16B). As expected the MEK inhibitor PD98059 (200nM) dramatically reduced Erk activation. Our data indicate that the basal levels of Erk activation present in motoneurons might be mediated in part by an autocrine and/or paracrine effect of endogenous BDNF but was mainly neurotrophin-independent.

2.4 DISCUSSION

The results of this study demonstrate for the first time that intracellular cAMP in embryonic motoneurons is essential for neurite outgrowth and extension. Pharmacological reduction and elevation of cAMP levels inhibit and promote neurite outgrowth, respectively. Intracellular cAMP mediates its effects on axon outgrowth in motoneurons via activation of PKA but independent of the activation of the Ras-Erk pathway.

These are important results for several reasons. First they demonstrate in conjunction with evidence for the role of cAMP in axon outgrowth in sensory neurons in the PNS and the CNS, that elevation of cAMP and activation of PKA represent a

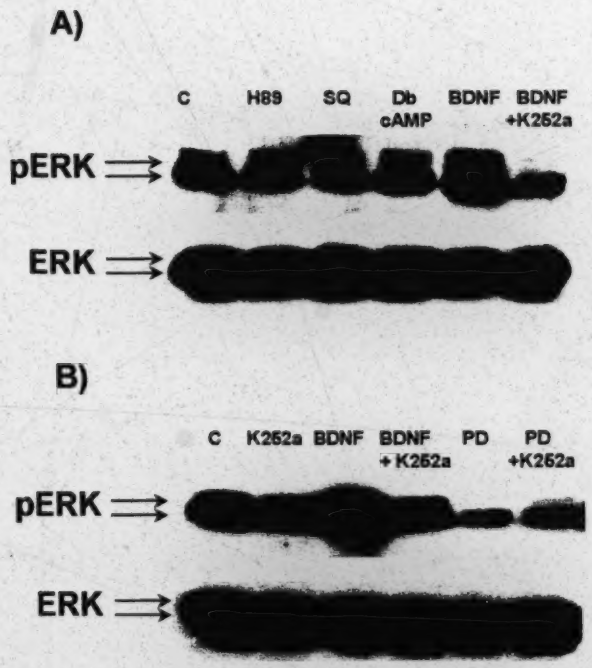

Figure 16. Cyclic AMP does not regulate Erk activation in motoneurons.
Motoneurons were cultured at a density of 30,000 per 24-well dish and treated at the time of plating for 2h with H89 (20 μM), SQ22356 (500μM), DbcAMP (1mM), BDNF (50 ng/ml), K252a (500 nM), BDNF (50 ng/ml) and K252a (500 nM), PD98059 (50 μM), PD98059 (50 μM) and K252a (500 nM). Equal amount of protein for each treatment was separated by SDS-PAGE and phosphorylation of Erk1/2 was examined by immunoblot analysis as described in the Materials and Methods. The figure is a representative of at least three independent experiments.

fundamental mechanism in axon outgrowth in the nervous system. Second, they provide evidence that the downstream pathways of cAMP-PKA that control gene transcription and in turn, axon outgrowth are not common to all neurons. This is consistent with the many downstream targets for PKA and with the differential control of transcription of the growth associated genes for axon outgrowth (Andersen and Schreyer, 1999; Bradbury et al., 2000; Teng and Tang, 2006).

2.4.1 Cyclic AMP promotes neurite outgrowth in sensory and motoneurons

The promotion of neurite outgrowth by cAMP has been demonstrated in DRG neurons which extend axons in both the CNS and the PNS. Our results establish that cAMP also promotes neurite outgrowth of PNS motoneurons on a permissive substrate indicating a common mechanism for both the motoneurons and the DRG neurons. The high levels of cAMP in DRG neurons during development are important for the growth of the DRG neural crest-derived sensory neurons into the spinal cord and for the establishment of connections with interneurons at different spinal cord segments and cranial-directed axons in the dorsal columns (Cai and Filbin, 1999; Neumann and Woolf, 1999; Cai et al., 2001; Neumann et al., 2002). When cAMP levels decline after birth, DRG axons can regenerate after nerve injury in the permissive Schwann cells environment of the PNS but not in the inhibitory environment of the CNS unless cAMP is elevated (Cai and Filbin, 1999; Cai et al., 2001). A conditioning lesion of DRG neurons in which the peripheral axons are injured by crushing before or at the same time as the central axons, promotes the regeneration of the central axons in the dorsal column synchronous with elevation of cAMP in the neurons (Neumann and Woolf, 1999).

Even though both sensory DRG neurons and motoneurons regenerate their axons after peripheral nerve injuries, functional recovery is generally poor because of a time-limited capacity of injured sensory and motor neurons to regenerate and for Schwann cells to support their axon regeneration in the denervated distal nerve stumps (Fu and Gordon, 1995b, 1995a, 1997). The protracted time period of outgrowth of axons from the proximal nerve stump of cut peripheral nerves that may be as long as 4 weeks in the rat, is rate limiting for axon regeneration and in turn, for functional recovery (Brushart et al., 2002; Gordon et al., 2003). Electrical stimulation greatly accelerates this axon outgrowth (Al-Majed et al., 2000; Brushart et al., 2002) and recent investigation of subcutaneous delivery of rolipram in concentrations that promoted regeneration of axons in the CNS, indicate that the rolipram, like the electrical stimulation accelerates this neurite outgrowth of both DRG and motoneurons in the PNS (Nikulina et al., 2004a; Nikulina et al., 2004b). Our demonstration of the critical link between elevation or depression of cAMP levels in motoneurons *in vitro* with promotion or reduction of neurite outgrowth and extension respectively provides the first direct evidence that cAMP levels control neurite outgrowth of motoneurons. This is strong evidence for a role of cAMP in axonal regeneration in the PNS.

The findings also indicate that procedures that elevate intracellular cAMP levels accelerate axon regeneration by promoting both the outgrowth of axons from the proximal nerve stump as well as by accelerating the rate of axonal growth. Such a procedure is the conditioning peripheral nerve crush lesion *in vivo* that either proceeds or occurs simultaneously with the injury and repair of the nerve (Jacob and McQuarrie, 1993; Neumann and Woolf, 1999; Torigoe et al., 1999; Neumann et al., 2002) The

conditioning lesion accelerates the rate of slow axonal transport and, in turn the rate of axonal regeneration (Jacob and McQuarrie, 1993). The brief period of electrical stimulation that promotes axon outgrowth of DRG and motoneurons does not effect the rate of axon regeneration however (Brushart et al., 2002; Al-Majed et al., 2004). Yet, the same electrical stimulation of the intact peripheral axons of DRG neurons whose central axons are cut in the spinal cord elevates cAMP in the neurons and in turn, promotes axon regeneration of the cut central axons as does the conditioning lesion (*unpublished observation-Furey and Gordon, 2006*). Hence elevated cAMP is essential for axonal regeneration in both the CNS and the PNS.

2.4.2 Cyclic AMP mediates its effects via PKA in motoneurons

Our results show that cAMP mediates its effects on neurite outgrowth via PKA activation in motoneurons. This is the same mediator of cAMP previously demonstrated in several neuronal types including DRG, cerebellar and retinal ganglion neurons (Cai et al., 2001). Nevertheless some differences exist between the motoneurons and the other neuronal types. Newborn DRG neurons (P1) extend neurites both on permissive and non-permissive substrate, however, only neurite outgrowth on a non-permissive substrate is blocked by PKA inhibitors and cAMP antagonists (Cai et al., 2001). Our results in contrast demonstrate that PKA downstream of cAMP regulates neurite outgrowth and extension of motoneurons on a permissive substrate and by analogy, may regulate motor axonal regeneration in the PNS. As yet, we have not established the downstream pathway(s) of cAMP/PKA that mediate axon outgrowth and extension in the motoneurons.

Downstream of PKA it has been established that activation of the transcription factor CREB is sufficient to remove MAG inhibition and promote neurite outgrowth in DRG neurons (Gao et al., 2004). Neurite extension of cerebellar neurons cultured on permissive substrate in contrast to neurons cultured on the inhibitory substrate of CNS myelin and/or MAG was not affected by the transcription inhibitor 5, 6 dichloro-1-b-D-ribo-furanosyl-benzimidazole (DRB) (Cai et al., 2002) suggesting that neurite extension and elongation on a permissive substrate does not require gene transcription at least in the first 18h. Among the identified targets of PKA important in neurite extension on a non permissive substrate, the enzyme Arginase 1 (Arg 1) is involved in the PKA-mediated reversion of DRG neurite outgrowth inhibition by MAG and myelin. Overexpression of Arg 1 in cerebellar neurons is sufficient to overcome inhibition by MAG and myelin however it does not affect neurite outgrowth on a permissive substrate (Cai et al., 2002). This suggested that activation of Arg I represents a mechanism to overcome neurite growth inhibition. We are yet to identify the downstream mediators of PKA in neurite outgrowth in motoneurons; these may or may not be the same pathway(s) previously discovered.

2.4.3 Erk is not activated downstream of cAMP in motoneurons

The Ras-Erk is a well documented signaling pathway that regulates cell proliferation, differentiation and growth in many cell types including neurons (Stork and Schmitt, 2002; Pouyssegur and Lenormand, 2003; Dumaz and Marais, 2005). This pathway regulates cAMP-mediated neurite outgrowth in PKA-dependent and PKA-independent ways in neurons and neuronal cell lines (Charles et al., 2003a; Gao et al.,

2004; Chu et al., 2006). Intracellular cAMP leads to Erk activation in DRG neurons and in turn, activation of transcription factors that include CREB. In motoneurons we demonstrate that cAMP does not activate Erk whether or not CREB is activated by the cAMP. Hence Erk phosphorylation is not an essential component of the effectiveness of cAMP in promoting axon growth in motoneurons in the PNS. Moreover inhibition of PKA with H89 does not affect Erk phosphorylation. This shows that cAMP/PKA does not mediate neurite outgrowth via the Ras-Erk pathway in motoneurons in the PNS as it does for sensory axon growth in the CNS and the PNS. On the other hand treatment of motoneurons with BDNF resulted in Erk activation as expected and might mediate neurotrophin-induced growth in motoneurons. The transcription factors and the genes activated in Erk-independent pathway might or might not differ from those activated in DRG and cerebellar neurons.

2.5 Conclusion

In summary we have demonstrated that cAMP mediates neurite outgrowth through PKA in motoneurons. The cAMP and PKA effects are not mediated via Erk, therefore it will be important to determine whether CREB represents the main transcription factor in motoneurons as it does in DRG and cerebellar neurons or whether other transcription factors play more important role in mediating neurite outgrowth effect of cAMP. Therefore understanding the molecular mediators of cAMP signaling in neurite outgrowth in motoneurons would be very helpful in designing studies to elucidate the role if any of cAMP and its downstream mediator PKA in accelerating axonal

regeneration after injury in motoneurons which processes are often slow and associated with poor functional outcomes.

CHAPTER 3

GENERAL DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

3.1 General discussion and conclusions

Of the many neuronal systems studied, motoneurons have been minimally investigated as far as the effect of cAMP on regeneration is concerned. It is known that neurons of the PNS regenerate their axons spontaneously after injury but the regeneration observed in some cases has been slow and staggered (Fu and Gordon, 1997). An attempt by Carlsen to elucidate the effects of cAMP on PNS axonal regeneration has been neglected since the 80's. Using a cell culture model we are able to molecularly explain to an extent the mechanisms by which cAMP mediates neurite outgrowth in motoneurons. Our studies have established the effect of cAMP on neuritogenesis and neurite extension in motoneurons in culture. To promote neurite outgrowth in motoneurons, cAMP could be elevated via different mechanisms including the application of the permeable cAMP analogs, use of physiological means of generating cAMP for example the use of forskolin and by pharmacological inhibition of the phosphodiesterases that degrade cAMP. The effectiveness of these approaches of intracellular cAMP elevation provides variety of ways to effectively elucidate mechanism of cAMP effects on neurite outgrowth in culture. Such studies often present difficulties under *in vivo* conditions. In addition the various pharmacologic agents used in these studies are comparatively safer *in vitro* and dose effects determination easier which is a problem with *in vivo* studies. For example in *in vivo* studies Rolipram presents with problems of nausea and vomiting leading to weight loss in experimental animals.

It has been established here that the main downstream target by which cAMP mediates neuritogenesis and neurite extension in motoneurons is through PKA. The exclusion of the involvement of Erk in the pathway downstream of either cAMP or PKA

is compelling as in DRGs and many neurons; Erk is activated in response to cAMP elevation with Erk being implicated in axonal outgrowth. In their study, Filbin and her group have shown that a novel cross talk between the two signaling pathways (cAMP and Erk signaling pathways) account for the ability of neurotrophins to elevate cAMP levels in neurons and to overcome MAG and myelin inhibition and in turn promote axonal regeneration in the CNS. They have also shown Erk activation when the DRG neurons were treated with the cAMP agonist dbcAMP (Gao et al, 2003; Cai et al, 1999)..

The pathway by which cAMP mediates neurite outgrowth in motoneurons as determined by our studies is represented in Fig 17.

3.2 Future directions

3.2.1 The role of polyamines in neurite outgrowth in motoneurons

It is important to investigate whether elevating cAMP in motoneurons result in increased expression and subsequent synthesis of polyamines and if they have any effect in promotion of neurite outgrowth. In DRG neurons cultured on non-permissive substrates the gene for Arginase 1 is expressed with subsequent synthesis of the polyamines that removed myelin/MAG inhibition and promoted neurite outgrowth. Polyamines on their own promoted neurite outgrowth and axonal regeneration in variety of neurons. Exploring this avenue in motoneurons will have much significance in nerve regeneration *in vivo* as it is known that polyamines accelerated facial nerve regeneration after injury (Gilad et al., 1996) which may lead to the understanding of the end effect of cAMP elevation on neurite outgrowth and axonal regeneration.

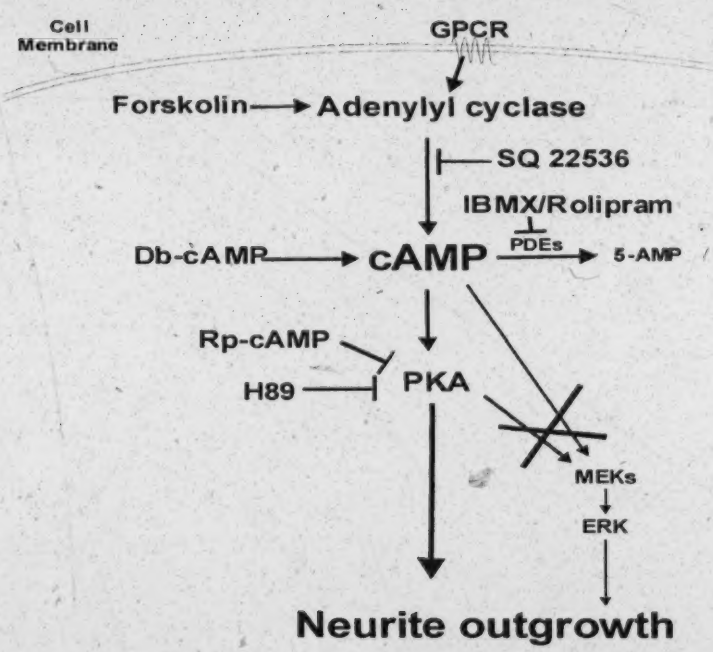

Figure 17. Diagram to illustrate cAMP signaling pathway responsible for promotion of neurite outgrowth in cultured motoneurons. Through G-protein couple receptor activation,, adenylyl cyclase converts ATP to cAMP which activates PKA to promote neurite outgrowth in motoneurons. Forskolin treatment raised cAMP and promoted neurite outgrowth. Conversely SQ22356 reduced intracellular cAMP levels and reduced neurite outgrowth. Prevention of cAMP degradation by the cAMP phosphodiesterases into 5'AMP by rolipram and IBMX raised intracellular cAMP and promoted neurite outgrowth. Neurite outgrowth in response to raised intracellular cAMP was mediated through PKA which inhibition by H89 or RpcAMP resulted in reduction in neurite outgrowth. Erk though known to be involved in neurite outgrowth and survival did not show a marked activation in response to elevated cAMP and might not be involved in cAMP mediated neurite outgrowth in motoneurons.

3.2.2 Receptors coupled to Gs-protein

decrease intracellular cAMP levels and consequently the reduction in neuritogenesis and neurite extension and the fact that stimulation of the adenylyl cyclases with forskolin raised intracellular cAMP and promoted neurite outgrowth, it would be significant to investigate which G-protein coupled receptors are involved in the generation of cAMP and the promotion of neurite outgrowth in motoneurons. Further study to determine the expression of such receptors in motoneurons and expression studies on neurite outgrowth would open further avenues to explore in understanding the cellular and molecular mechanisms of neurite outgrowth in culture and axonal regeneration *in vivo* which would serve as targets for the development of chemical entities that could be pharmacologically used in place of forskolin. One such group of receptors implicated in neurite outgrowth is the cAMP coupled-adenosine A2A receptors (Charles et al., 2003b)

3.2.3 Culture systems to investigate cell body and axonal mechanisms of cAMP effects

There is evidence that the effects of cAMP on neurite outgrowth in certain groups of neurons for example DRG neurons are mediated at the level of the cell body (Chierzi et al., 2005). Culturing motoneurons in compartment cultures could help elucidate cell body and axonal mechanism involved that otherwise presents with difficulty under the current motoneuron culture model. Compartment culture model has been extensively used to investigate and differentiate axonal from cell body mechanisms in the rat sympathetic ganglia including lipid metabolism and transport (Vance et al., 1995; de Chaves et al., 1997), and ceramide metabolism and neuronal survival (Song and Posse de Chaves,

2003). Such a study with motoneurons in compartment cultures would be very insightful and could be made to simulate *in vivo* conditions of axonal injury and regeneration. The focus of this experiment has largely been what happens within the first 24 hours in culture (short term culture model). It will be significant as well to know what happens later for example in long term cultures for up to a period of two weeks. Compartment cultures with conditions set to simulate *in vivo* conditions could help shed more light on later molecular events both at the level of the cell body and the axons.

3.2.4 Cyclic AMP and Erk activation in motoneurons

We have shown that Erk is not activated in response to elevated cAMP under the conditions of the experiments and may not be involved in cAMP mediated neurite outgrowth in motoneurons. This study on Erk activation by cAMP needs to be further investigated at different time points during the time for neurite outgrowth in the motoneurons. It is also recommended that in future experiments be done to investigate if Erk activation under basal conditions as observed for control cultures has any role in neurite outgrowth in motoneurons. Treatment of the cultures with BDNF elicited marked activation of Erk. It will be worthwhile investigating if this marked Erk activation in turn inhibits cAMP phosphodiesterases with a consequence of elevated cAMP as observed with DRG neurons cultured on non-permissive substrates.

4.0 References

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